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Selig Hecht

The photograph of Dr. Hecht was taken by Dr. Simon Shlaer

SELIG HECHT

(1892-1947)

By GEORGE WALD

On last September 18, Selig Hecht, Professor of Biophysics at Columbia University, died suddenly at the age of 55. He had been one of the most vivid scientific figures of his time; a pioneer in the development of general physiology in this country; and for more than two decades leader in his chosen field, the physiology of vision.

In Hecht, great scientific capacities were combined with superb gifts as a teacher, writer, and lecturer. His interests ranged widely, and everywhere they touched he made some striking personal contribution. The world will miss his vigorous personality, his breadth of outlook, and his generosity of spirit no less than his works.

Into his special field, Hecht had instilled something of his own clarity, substance, and force. He drew together its scattered phenomena, rationalized them, and gave them a secure foundation in physics and chemistry. His work and that of his laboratory have contributed in many areas of vision the most complete and accurate data that we possess. They have also provided a context of ideas and rigorous theory upon which workers in vision will rely for many years to come.

Hecht cast his light widely, and many found their way by it. In his death, his colleagues recognize the passing of a great scientist; and they and many others feel the loss of a warm friend.

I

Selig Hecht was brought to America as a young child from his birthplace in the village of Glogow, then Austrian Poland. It was the period of the great migration to this country from Eastern Europe, and followed a pattern that one can recapture now only in such accounts as Mary Antin's "Promised Land." The family settled in New York's lower East Side, and there young Selig went to public and Hebrew schools, and was taught Hebrew at home by his father.

The early history of the family is filled with struggle and difficulty. Selig was the eldest of five children; and the boy ran errands after school to add to the Hechts' small resources. Later, at high school, he found work as book-keeper in a woolen business, a position he kept all through college. The ideal of learning under difficulties was deeply embedded in the family's outlook.

Selig's father himself turned to serious study as soon as he could win some leisure, and now 80 and still vigorous, reads widely and argues warmly through problems in history and philosophy, Schopenhauer and Spinoza.

Selig entered the College of the City of New York in 1909, and there began to concentrate in mathematics. It was only late in college that he took a first course in zoology, and turned to this as his primary interest. The summer vacation before leaving college Hecht spent with his fellow student William Crozier on a fellowship at the Bureau of Fisheries Station at Beaufort, North Carolina. Out of the summer's work came two papers—one written jointly with Crozier—on the relation of weight to length in fishes; and one on the absorption of calcium during molting of the blue crab.

On graduating in February, 1913, Selig went to work as chemist in a fermentation research laboratory. Here he made his first contact with photochemistry, having been asked to study the effect of light on the deterioration of beer. On solving this problem, he was promptly discharged. He determined then to renounce industrial work forever.

Back at Beaufort for another summer, Hecht made plans to begin graduate study. To obtain funds for this he took a position as chemist in the Department of Agriculture in Washington. Within a year he had saved enough to enter Harvard for graduate training in zoology.

At Harvard Hecht became one of a group of graduate students who were to play a major rôle in the development of general physiology in this country—Crozier, Fenn, Redfield, S. C. Brooks, Olmstead, and Minnick. He undertook research for the doctorate under G. H. Parker, and studied also with Osterhout, Wheeler, Mark, and Rand. Summers were spent at the Bermuda Biological Station, working on the physiology of *Ascidia atra*, the subject of his doctoral dissertation.

The Ph.D. was granted in June, 1917; and on the following day Selig was married to Celia Huebschman, daughter of an immigrant Austrian family, whom Selig had met while at college in New York. It is difficult to think of either person thereafter without the other. They shared an extraordinary community of interest and enjoyment; and dealt with each other on an intellectual level to which few marriages attain. Wherever they were, Celia made a home warm with hospitality and grace, to which Selig could bring his friends and his troubles, sure that both would be received with sympathy and understanding.

Their wedding was brightened by a characteristic incident. Selig had entered a portion of his doctoral thesis for the Bowdoin Prize "for essays of high literary merit," and was awarded two hundred dollars and a medal. With this puff to their fortunes the young couple left for a honeymoon at the Oceanographic Institute at La Jolla. Ritter, the director of the Institute, gave Selig a fellowship for the summer. It was under these circumstances that

he performed the experiments on the sensitivity to light of the ascidian, *Ciona*, which launched a lifetime of work on photoreception and vision.

The paper which described this investigation and presented for the first time Hecht's view of the photoreceptor process was sent to Jacques Loeb for publication in the newly founded *Journal of General Physiology*. It appeared in the first volume. From then on Hecht's entire scientific production, with only minor exceptions, was published in the pages of this *Journal*. Though he never played a formal rôle in its direction, Hecht felt identified with its purposes and standards, and never failed to send it the best of his achievements in their most definitive form.

In the fall of 1917 Selig took the position of Assistant Professor of Biochemistry in the Medical School of Creighton University, a Jesuit institution in Omaha. Here he spent the next four years.

Hecht was made for the metropolis and it for him; and he could scarcely look upon this period otherwise than as a species of exile, made more onerous by lack of time and resources for research. Each summer he spent in Woods Hole, eagerly compensating for the year's frustrations. Here at this time he did some of his most significant work. The analysis of photoreception, introduced with the *Ciona* experiments, was now worked through in detail with another relatively simple system, that of the clam *Mya*. As his theory became more firmly established, and Hecht grew more confident of its generality and essential correctness, he turned to the analysis of a human visual function, dark adaptation. Seeking also for direct information on the initial effects of light on the eye, he now performed his classic studies of the bleaching of rhodopsin in solution.

Selig was now wholly caught up in what he believed to be a major scientific development. He wanted to establish an adequate laboratory, to teach general physiology which for him was an area of science endowed with a mission, and to develop about himself a group of research students. That no opportunity was made available to do these things was without question a source of deep disappointment.

With Jacques Loeb's sponsorship, Selig was awarded a National Research Council Fellowship in Biology, which he held for three years. Then, with no post in sight, he was appointed for another two years to a General Education Board Fellowship. This was a trying period, though rich in experience and in the further development of his work. It is a strange thing that Hecht, for all his gifts and superb accomplishment, waited almost a decade after completing his formal training before receiving his first adequate academic appointment.

The warm friendship and confidence of Jacques Loeb, whom he had come to know at Woods Hole, was a continuing source of encouragement. In a letter in the fall of 1922 Loeb wrote him: "I feel that in you the coming generation of scientists will have a leader and that I need not yield to my pessimistic

moods in regard to the future of science in this country. You yourself may safely ignore the stupidity and even brutality of our times and keep that serenity which is required of a man who wishes to do his best work. The future needs you and belongs to you." Then, in a characteristically gracious footnote, "Please remember me kindly to Mrs. Hecht—she may well be proud of you."

Hecht spent his first year as National Research Council Fellow in the laboratory of the photochemist E. C. C. Baly, in Liverpool. There, with the help of R. E. Williams, he carried through a classic study of the spectral sensitivity of human rod vision, providing measurements of this function which have not since been adequately superseded.

The remaining two years of the Fellowship were spent as a guest in the laboratory of L. J. Henderson at Harvard Medical School, and at Woods Hole. During this period Hecht extended his view of the photoreceptor process to a theoretical analysis of brightness discrimination, characteristically embracing the data for man and for the clam in one quantitative treatment. Here he pointed out for the first time that the data of human intensity discrimination are dual in origin, breaking on analysis into a low intensity portion dependent on the rods, and a high intensity segment governed by the cones.

In the spring of 1924 the Hechts' daughter, Maressa, was born. The family spent the following year at Naples, where Selig, as a General Education Board Fellow at the Zoological Station, worked on *Ciona*, and on a new lamelibranch, *Pholas*.

The following year the Hechts lived in Cambridge, England, where Selig entered Barcroft's laboratory. One could hardly do this without being drawn into the lively controversy that revolved about the question whether the oxygen dissociation curve of hemoglobin is S-shaped or a hyperbola. Selig, for all his absorption in visual problems, plunged into this work, and devised a spectrographic procedure, of which Barcroft in his "Hemoglobin" (p. 158) wrote, "This technique, so far as the making of all the estimations is concerned, is in many ways a decided advance on any of its predecessors. The improvement was aptly expressed by someone who, looking at one of Hecht and Morgan's curves, said, 'This is the first dissociation curve I have seen where the points really lie on the curve.'" In spite of this gain the curve continued to vacillate unaccountably between an S and a hyperbola, then and for several years afterward.

The year in Cambridge was the last of Hecht's *Wanderjahre*. During these fellowship years, with their opportunities for visiting and travel, and at the Physiological Congress in Stockholm in 1926, the Hechts formed many warm friendships abroad, which they maintained and cherished ever afterward, and renewed at every opportunity. In this period also Hecht gained a wide international audience for his work. A general review which he wrote for *Natur-*

wissenschaften in 1925 led to a published discussion with Lasareff. In the years that followed, stemming still in part from associations formed in this earlier period, Hecht wrote again in *Naturwissenschaften* (1930); a comprehensive review in the Asher-Spiro *Ergebnisse der Physiologie*, translated into German by Frau Asher (1931); a volume in the *Actualités Scientifiques* (1938); and invited papers in several British journals.

In the spring of 1926 Selig was offered simultaneously a post at Columbia and a projected chair at a major English university. Much as he had valued his English associations, he decided to return to this country; and in September, 1926, he became Associate Professor, in 1928 Professor of Biophysics at Columbia University, the post he held until his death.

II

At Columbia, as the only physiologist in the Department of Zoology, and with a large measure of autonomy within his special sphere, Hecht could construct a situation after his own design. In lofty isolation on the thirteenth floor of the new Physics Building, commanding a superb view of the city to the southward and of the Hudson River, he designed a compact group of laboratories and workrooms that contained everything needed for physiological investigation and instruction. Here he began to give an advanced course in general physiology, in which he spread before a small and well prepared group of students his highly original ordering of the subject. The students were given individual problems in the laboratory, and most of the initial group remained to complete with him their doctoral researches.

Hecht took a quite extraordinary interest in his students. The layout of the laboratory itself encouraged association. Tea was served every afternoon, and here, and at weekly colloquia, indeed on any occasion in which Hecht or one of the students had something he wished to discuss, a group would gather. Conversation and argument ranged over wide areas, in and out of science: literature, politics, music, and art. For a period the students met for an evening each week at the Hechts' home to read and discuss L. J. Henderson's "Blood" and P. W. Bridgman's "Logic of Modern Physics" as each of these books appeared. Later in the evening Mrs. Hecht would join the group over sandwiches and beer, and the conversation would broaden its scope. This communal life of the laboratory articulated and clothed the bare bones of graduate instruction. It fostered in Hecht's students a strong and abiding sense of attachment and loyalty. In after years Hecht and the laboratory continued to hold a central place in their thoughts and affections.

Among the first students to enter the laboratory was Simon Shlaer, who became Hecht's assistant in his first year at Columbia, and continued as his associate for twenty years thereafter. Shlaer, a man infinitely patient with things and relatively impatient with persons, gave Hecht his entire devotion.

He was a master of instrumentation, and though he had also a very keen grasp of theory, devoted himself by choice to the development of new technical devices. Hecht and Shlaer built a succession of precise instruments for visual measurement, among them an adaptometer and an anomaloscope which have gone into general use. The entire laboratory came to rely upon Shlaer's ingenuity and skill; and after he left in 1947 Hecht remarked, "I am like a man who has lost his right arm and his right leg."

In the Columbia laboratory Hecht instituted investigations of human dark adaptation, brightness discrimination, visual acuity, the visual response to flickered light, the mechanism of the visual threshold, and normal and anomalous color vision. Important contributions were made also to the biochemistry of visual pigments, human vitamin A deficiency and night blindness, the spectral sensitivities of man and other animals, and the light reactions of plants: phototropism, photosynthesis, and chlorophyll formation.

The Columbia laboratory under his direction became one of the most productive centers of physiological investigation and training, and Hecht himself exercised an ever-widening influence and activity in contemporary science. Almost a score of his students left the laboratory to pursue careers in physical and biological chemistry, physiology, chemical genetics, and ophthalmology. Hecht was awarded the Frederick Ives Medal of the Optical Society of America in 1941; and was elected to the National Academy of Sciences in 1944. He was a Director-at-large of the Optical Society of America, and served on the Editorial Boards of the *Journal of the Optical Society*, the *Biological Bulletin*, and *Documenta Ophthalmologica*.

Throughout the late war Hecht devoted his energies and the resources of his laboratory to military problems. He and Shlaer developed a special adaptometer for night-vision testing which was adopted as standard equipment by several Allied military services. Hecht directed a number of visual projects for the Army and Navy, and served as consultant and advisor in many others. He was a member of the National Research Council Committee on Visual Problems, and of the Executive Board of the Army-Navy-OSRD Vision Committee.

His influence, however, extended far beyond the scope of these formal commitments. He visited many military installations to acquaint himself at first hand with their problems; and took researches into the field whenever it seemed that that might help to bring quicker or more practical solutions. He had a strong sense of the urgency of the war, and no civilian timidity whatever. His plain speech in high places won the esteem and affection of his military associates. They miss him as deeply as do his academic colleagues.

Hecht had a high sense of the social obligations of science. He thought it imperative that science be explained to the layman in terms that he could understand and could use in coming to his own decisions. For this task he

himself had a special talent. He greatly enjoyed giving a number of courses for adults at the New School, on sensory physiology, physics, and atomic energy. When it appeared to him that the lay public was being misled by statements from certain of his colleagues involving Heisenberg's indeterminacy principle with the problem of human free will, he wrote an essay on the subject for *Harper's Magazine* (1195). Early in the War he wrote for *Harper's* also an article on night vision, which was distributed later in large numbers to the Air Force.

Hecht's lectures on atomic energy at the New School grew into his book "Explaining the Atom," a lay approach to atomic theory and its recent developments, called in a *New York Times* editorial (September 20, 1947) "by far the best so far written for the multitude." Hecht was deeply concerned with the effort to abolish the military uses of atomic energy and to turn it toward constructive ends. He was honorary vice-president of the Emergency Committee of Atomic Scientists, the only member of this small group who was not a nuclear physicist.

His book had one curious consequence; he was asked to lecture on atomic energy before the War College. He accepted the invitation, but characteristically changed the subject. He lectured instead on the relation of science to technology, pointing out the need, now that the war had ended, to foster the basic scientific research upon which all technology depends.

III

A man's work merits a biography of its own. It has its own ancestry, birth, and development; its own span of life. Selig Hecht's work was a particularly vigorous growth, and it will long survive him.

All is grist to a mind as original as Hecht's, yet several early influences, going back to his graduate years at Harvard, made a particular impression. He spoke of them often afterward, and they are apparent in his work throughout a long period. One is the nascent science of photochemistry, coming to fruition in the first decades of the century in the laboratories of Luther and his colleagues Weigert and Plotnikow. Another is Jacques Loeb's treatment of animal phototropism; his generalization of its fundamental mechanisms to include both animals and plants, and his insistence that phototropic excitation has its source in ordinary physicochemical processes. The third influence complemented the others. It was Arrhenius's book, "Quantitative Laws in Biological Chemistry," published in 1915. Hecht later mentioned the excitement with which he and some of his fellow students at Harvard received it. It held out the promise that by accurately measuring biological functions, and fitting to them the simple equations of chemical kinetics, one could reveal their underlying physicochemical mechanisms.

Hecht launched his attack on photoreception with an intensive study of

such relatively simple, unorganized systems as are associated with light reflexes in the ascidian *Ciona* and the clam *Mya*. These are highly manipulable organisms, their responses definite, their reactions slow enough to be measured without elaborate apparatus, their temperatures susceptible to wide variation. All these virtues appear to full advantage in Hecht's experiments.

Out of these researches came the concept of the photoreceptor process as a reversible—or more properly pseudoreversible—system, in which a photosensitive pigment is attacked by light and is simultaneously restored by ordinary thermal reactions. In the light, the concentration of photopigment declines to some constant, steady-state value; in darkness it is restored to a maximum level. In these processes Hecht recognized the chemical sources of light and dark adaptation.

The steady state achieved under a constant illumination has significant properties of its own. The simple animals with which Hecht began his work respond to *changes* in illumination; in the steady state they behave as though the light no longer stimulates them. In this sense the light-adapted state resembles the dark-adapted condition. Both provide a constant background upon which a stimulus can be superimposed: an absolute threshold upon the dark-adapted state, a differential threshold upon the light-adapted state. Hecht had written equations for the steady state; and by assuming that the visual threshold, absolute or differential, corresponds to a constant increment in the rate of breakdown of photosensitive material, he could extend his treatment to departures from the steady state—the phenomena encountered in brightness discrimination, the response to flickering light, and the absolute threshold.

This theoretical apparatus, based upon his study of invertebrate systems, Hecht turned without important modification to the examination of human vision. His contribution here is the most comprehensive since Helmholtz. Yet he never ceased to test the general validity of his ideas in the dialectic of organic evolution. Dark adaptation was explored in man, the molluscs *Mya* and *Pholas*, and the tunicate *Ciona*; visual acuity in man and in the compound eyes of the bee and fruitfly; intensity discrimination in man, *Mya*, *Pholas*, *Ciona*, and the fruitfly; flicker in man and the clam.

What particularly distinguished Hecht's contribution from earlier, general statements on the nature of light reception in animals and plants, was its breadth and perseverance, and its insistence on definiteness and rigor. At every turn Hecht developed his theories in mathematical form, and produced accurate measurements to test them. Always he laid emphasis on the maximum simplicity of theory that could be reconciled with maximum concreteness. He believed it more important that a theory be definite and that it convey an illuminating idea than that it attempt at once to cope with all foreseeable complexities.

Another important quality distinguished Hecht's contributions. They were presented in striking and convincing fashion, often in several versions designed to expose their various facets. Hecht's papers were worked and reworked. They were models of careful design, written in a vigorous prose spiced with graphic and telling phrases, each word chosen to convey the author's intention to the reader. Hecht drew and lettered all his own figures, and approached their composition with all the care he devoted to one of his paintings. His scientific lectures had the same qualities of design and persuasion. He had the gift of seeming to enter into close and earnest communion with even large audiences. When he had convinced himself of the reality and fruitfulness of an idea, he taught it unforgettably to his generation.

At Columbia, Hecht set a rigorous standard for the work of his laboratory. He was imbued with the ideal of the "classic experiment," one done so thoroughly and well that it should never have to be repeated.

This technical ideal, however, was subordinated to intellectual ends. Hecht had an unequalled grasp of the literature of his field, and worked with it constantly, drawing it together, rationalizing it, recalculating, cutting and fitting; attempting to achieve through this process an integrated view and a guide to fruitful experiment. Before he started any experiments in human dark adaptation, visual acuity, intensity discrimination, or color vision, he had already published theoretical approaches to these functions on the basis of existing data. Having repeatedly been frustrated by incomplete or inadequate information, he was determined that measurements from his own laboratory should be precise and exhaustive.

In 1929, at the Thomas Young Centenary celebration at Cornell University, Hecht presented a brilliantly original synthesis of what had been the unorganized quantitative data of human color vision, the first attempt to provide a reasonably comprehensive theory in this field. Starting from the heritage of trichromatic theory propounded by Young, Helmholtz, and Maxwell, Hecht took this to imply the existence of three types of cones, and attempted to define their characteristics and physiological interrelations. The most distinctive outcome of his analysis was that the sensitivities of all the cones must lie very close together in the spectrum. In this his theory differs sharply from all previous formulations.

Hecht derived spectral sensitivity functions for the three types of cones on the more or less arbitrary assumption that all three types make equal contributions to the brightness of white light. The last investigation in which he took part, however,—a comparison of the brightness function in normal and colorblind subjects—led to another conclusion. It seemed to show that each type of cone makes a different contribution to brightness, the "red" group the largest, the "blue" the least. Hecht had looked forward to exploring this possibility further.

Recently also Hecht had become intensely interested in quantum relations in vision. He and his colleagues had redetermined the minimum threshold of human rod vision, and in agreement with earlier measurements had found it to involve only 50 to 150 quanta of light. When all allowances had been made for surface reflections, the absorption of light by ocular tissues, and the absorption by rhodopsin which alone is effective in stimulation, it emerged that the minimum visual sensation corresponds to the absorption in the rods of at most 5 to 14 quanta. An entirely independent statistical argument led to the conclusion that the absolute threshold involves about 5 to 7 quanta. Both procedures agreed therefore in estimating the minimum visual stimulus at 5 to 14 quanta. Since the test field in which these measurements were performed contained about 500 rods, it is difficult to escape the conclusion that one rod is stimulated by a single quantum.

At the time of his death Hecht was in process of drawing the consequences of this fundamental discovery. He had become convinced that at all levels of illumination one has to deal with similarly small numbers of elementary events, and had prepared to modify many of his earlier theories accordingly.

Much has been made in the past of the varied reactivity of the organism in response to a constant stimulus. When, however, as in this case, the stimulus involves so few quanta, statistical variations in the stimulus become much more important than biological factors in varying the response. This new view also foreshadows a fundamental revision in the concept of the visual threshold; for this is bound up with the thought that there exists a lower intensity which fails to stimulate. In this sense a rod excited by a single quantum cannot be said to have a threshold, for no smaller amount of light exists than one quantum.

Hecht was also deeply interested in a general implication of this discovery for biology. Some question still persists whether biological systems are subject to the ordinary restrictions of thermodynamics; the most careful experiments have in general shown clearly that they are. Yet in the visual threshold one has a process which depends for its initiation upon so few quanta—and hence so few photopigment molecules—that it falls outside the province of thermodynamic treatment.

IV

Selig Hecht pursued his relaxations with all the wit and warmth that he did science. He understood music as do few non-professional musicians. He was a talented painter in water colors, and read widely and critically. To everything he did he brought unfailing zest and taste.

Wherever the creative faculty was at work, Hecht worked with it in spirit. He shared the problems of the composer at the symphony, the painter at the exhibition, the author of the book he read. Recognizing this for what it was, practitioners of all the arts dealt with him virtually as a colleague.

He took keen pleasure in all his activities and relationships, in science, painting, teaching, his life with his family, his friends and colleagues. He was the most genial of companions, witty, stimulating, sympathetic. He loved good conversation and fruitful argument. He was a warm friend; in fair weather and foul one could rely upon his understanding and help.

He asked of every aspect of his life the same qualities of taste and composition that he sought in science and painting. It is good to think that his life closed like a sonata movement recapitulating its main themes. In July he flew to England, and at the Physiological Congress at Oxford came together again with many old friends of earlier years. He went on to the Color Vision Conference in Cambridge, at which were gathered most of the workers in vision of Europe and America, and together with them, in a week of absorbing discussion and argument, thought through the status of his work, and laid plans for the years ahead. One afternoon in Cambridge he walked across the river to the house in which the Hechts had lived and he had painted in the garden twenty years before. He returned to America for the wedding of his daughter. Two weeks later he suddenly died, without long illness or apparent suffering.

With Hecht one always had the sense of wide spaces and a clear light. The world is smaller and dimmer for his going.

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A RAPID METHOD FOR THE DETERMINATION OF PROTEOLYTIC ACTIVITIES OF ENZYME PREPARATIONS

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The ability of an enzyme to liberate the titrable carboxyl groups from a protein substrate was used by Willstätter (1) as a measure of the activity of an enzyme. Anson (2) established a measure of enzyme activity through the determination of the digestion of denatured hemoglobin in terms of a phenol reagent and the blue color developed with reducing agents, such as tyrosine, tryptophane, etc. In both of these methods, the estimation of enzyme activity was carried out on the products of digestion. White and Bowman (3) believe that the estimation of the end-products of digestion need not be a true measure of the proteolytic enzymes present, since crude pancreatic extracts also contain peptidases. They therefore modified the Anson method of estimation of trypsin by measuring, before and after digestion, the undigested proteins with the phenol reagent. This method likewise requires a preliminary separation between the digested and undigested proteins. The objection raised by White and Bowman is not necessarily valid since the phenol color of the non-protein digestive products changes very little if at all on further digestion. However, it is likely that the high concentration of urea used to keep the hemoglobin in solution will also inactivate some enzymes (4).

A simple and rapid method for the estimation of the proteolytic activities of enzymes has emerged from the present study. This method is based on the determination of the amount of undigested protein substrate, which can be precipitated and determined rapidly and quantitatively by means of a turbidimeter. Rona and Kleinmann (5) described a similar nephelometric method for the estimation of tryptic activity by mixing a solution of sodium caseinate with a certain amount of enzyme. Samples were removed after different intervals of incubation and the undigested protein was precipitated and determined nephelometrically. We have chosen to measure the proteolytic activity by determining the amount of enzymes or the dilution of enzyme which under controlled conditions will digest casein to an extent wherein 50 per cent of the protein is no longer precipitable by trichloroacetic acid. Such an amount of enzyme is arbitrarily defined as one unit.

Experimental Procedure

At low concentrations of enzyme the initial rate of a catalyzed reaction usually bears a linear relationship to the amount of enzyme added to any

given concentration of substrate, provided the latter is high. Northrop and his associates utilized this situation to determine the activity of pepsin (6). This relationship was examined in the case of casein and a single rather active proteolytic enzyme. Twenty ml. of various concentrations of a crystalline trypsin solution was added to 20 ml. of 0.25 per cent casein solution at pH = 7.6 and 37.5°C. Two ml. samples were then taken at 5, 10, 13, 15, 17, 20, 25, and 30 minutes. The amount of the undigested casein in each sample was precipitated with 3 ml. of a 5 per cent trichloroacetic acid solution. The turbidity, so produced, was determined in a Klett-Summerson photoelectric colorimeter and plotted against time. The slope of the curves (K), being

TABLE I

The Relationship between the Rate of Digestion of Casein and the Concentration of Enzyme Used

(C) Crystalline trypsin nitrogen/ml. <i>micrograms</i>	K	$K/(C)$
1.142	9.88	8.65
1.428	12.50	8.75
1.599	13.75	8.60
1.999	17.25	8.63
2.399	21.38	8.91
2.570	22.50	8.75
3.199	27.75	8.67

linear at the initial phase of the digestion, was then measured for each concentration of enzyme used, where K was expressed as the reciprocal of the amount of undigested casein (in terms of galvanometer reading) at the given time. In other words,

$$K = \frac{t_a - t_0}{S_0 - S_a},$$

where the t 's and S 's denote the time of incubation in minutes and the concentration of the substrate, casein, in grams per cent respectively. The subscripts 0 and a denote zero and a minutes of digestion. The results given in Table I demonstrate that as the concentration of the crystalline enzyme (C of column 1) increases, the rate of digestion (K of column 2) increases proportionately. The quantitative relationship between the rate and enzyme concentration can best be demonstrated by the constancy of the ratio of $K/(C)$ (column 3). This relationship was utilized in formulating the proce-

cedure for the determination of enzyme activity, the details of which are given below:

(a) *Preparation of Solutions.*

1. *5 Per Cent Trichloroacetic Acid Solution.*—Fifty gm. of trichloroacetic acid C.P. is dissolved in water and made to 1 liter.

2. *Stock 5 Per Cent Casein Solution.*—Twenty-five gm. crude casein (Borden's 453) is stirred in 400 ml. of water with gradual addition of about 4 ml. of 4 N NaOH, until the protein is completely in solution. The solution is then adjusted to pH = 7.6 and made up to 500 ml. in a volumetric flask and the small residue removed by filtration through cheese cloth. Working units of this stock solution are kept frozen in a series of individual bottles.

3. *0.25 Per Cent Casein Solutions.*—Twenty-five ml. of the 5 per cent stock casein solution is pipetted into a 500 ml. volumetric flask containing 50 ml. of M/2 phosphate buffer at pH 7.6. The solution is made to volume with distilled water. Working solutions of the casein are made up freshly each day.

(b) *The Determination of Enzyme Activity.*

1. *Estimation of Turbidity Produced by Varying Amounts of Casein.*—To four colorimeter tubes are added 0.45, 0.50, 0.50, and 0.55 ml. of the casein solution. Enough 0.85 per cent saline solution is added to each tube so as to increase the volume to 2.0 ml. To these solutions is added 3.0 ml. of a 5 per cent trichloroacetic acid solution from a 25 ml. burette and the turbidities are measured in the Klett-Summerson photoelectric spectrophotometer *immediately and before* flocculation of the precipitate takes place. The average of the turbidity readings of the two duplicate samples of 0.50 ml. of casein is used as the value of 50 per cent digestion. Typical results from such an experiment are: 0.45 ml.—195; 0.50 ml.—210 and 205; 0.55 ml.—225. The readings of 0.45 ml. and 0.55 ml. of the casein solution are included to be certain that a 10 per cent variation in the casein precipitated by trichloroacetic acid has a measurable effect in turbidity.

2. *Estimation of a Dilution to Give Approximate Unit Activity.*—Much time can be saved if an approximate activity of an unknown enzyme solution can be estimated. Otherwise, the procedure of serial dilution as described in step 1 should be followed.

Step 1.—To a series of five tubes is added 1.0 ml. of 0.85 per cent NaCl solution. To the first tube is added 1 ml. of the enzyme solution of an unknown potency. After thorough mixing, 1 ml. of the solution in the first tube is carried over to the second tube, and 1 ml. of the content in the second tube is in turn carried over to the third tube. This process of serial dilution is repeated until the fifth tube is reached. One ml. of the content in this last tube

is then pipetted and discarded. One ml. of 0.25 per cent casein solution is then added from a 10 ml. burette to each one of the five tubes and incubated at 37.5°C. in a water bath for precisely 15 minutes. After incubation, 3.0 ml. of a 5 per cent trichloroacetic acid solution is added to the tube and the turbidities are measured immediately. If the dilution of the enzyme solution which contains one unit per ml. lies within the range of the serial dilution, the turbidity reading of the 50 per cent point will fall between the readings of two consecutive tubes. The original unknown enzyme solution should then be diluted to such an approximate concentration and be used for the final estimation of activity according to the procedure given in step 2.

Step 2.—To a series of nine tubes are added 1.0, 0.80, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, and 0.40 ml. of the unknown solutions diluted according to the preliminary findings of step 1 so that a unit of enzyme activity is contained in from 0.4 to 1.0 ml. A normal saline solution is again added so that each of these tubes contains 1.0 ml. of fluid. One ml. of 0.25 per cent casein is added to all tubes and incubated for 15 minutes, as described in step 1. The amount of undigested protein is precipitated with 3 ml. of 5 per cent trichloroacetic acid and the turbidity readings then made. From the turbidity readings, the dilution of enzyme which will give the 50 per cent point can be determined by interpolation. The enzyme contained in this dilution is then equal to one unit of activity.

Factors Affecting the Activity Determination

Since the rate of the digestion of casein can be affected by such factors as the concentration of casein and pH at a given temperature of incubation, it is important to ascertain the extent to which these factors must be controlled to insure the accuracy and reproducibility of the experiments.

(a) Concentration of Casein.

Since there is a quantitative relationship between the enzyme and the substrate, during the initial phase of digestion, it is important that the concentration of the casein be specified in order to define the unit activity. We have therefore determined the effect of varying the concentration of the casein solution on the activity determination by estimating the dilution of an enzyme solution (containing 333 units per ml.) which will digest 50 per cent of the casein solutions of different concentrations in 15 minutes at 37.5°C. at pH = 7.6. The results of such an experiment are given in Table II, demonstrating that as the concentration of the casein increased from 0.20 per cent to 0.30 per cent, the unit activity per milliliter found decreased. However, that this decrease bears a quantitative relationship to the casein concentration, is evident from the agreement between the units of activity per milliliter found, and those obtained by calculation. It is obvious from this that the concen-

tration of casein in the test need not be 0.25 per cent exactly, but if it is not, then the concentration should be known precisely so that a correction can be made in the final calculation of activity.

(b) *Effect of the pH on Digestion.*

Since the rate of digestion of casein by enzymes depends to a large degree on the pH of the medium, experiments were performed to ascertain whether small variation of pH will affect the determination of the enzyme activity. Hence,

TABLE II

The Effect of Concentration of the Substrate on the Unit of a Standard Enzyme Preparation

Casein used	Units activity per ml. (found)	Theoretical units* activity per ml.
<i>per cent</i>		
0.20	416	416
0.22	356	379
0.24	339	347
0.25	333	333
0.26	313	320
0.28	298	298
0.30	276	278

* Theoretical units activity per ml. = unit activity determined with 0.25 per cent casein multiplied by the ratio of caseins (0.25/the concentration of casein in column 1).

0.25 per cent casein solutions with pH ranging from 7.4 to 7.8 in the step of 1/10 of a pH unit were prepared and used as substrates. Our results demonstrate that within this pH range there appears to be no significant difference in the results of the determination.

(c) *The Effect of Different Preparations of Casein on the Enzyme Activity.*

It is well known that commercial preparations of casein are essentially a mixture of proteins. It is therefore of importance to ascertain whether different casein preparations can be used for the determination of enzyme activity. Experiments were performed to determine the activity of a crystalline trypsin solution containing 1.428 mg. N per ml. with several preparations of casein. The results tabulated in Table III demonstrate that the activity found is essentially the same regardless of the casein solutions used, provided they have been adjusted to the same concentration as measured by the turbidity readings rather than by the weight of the casein used. This satisfactory agreement of activity results led us to determine by electrophoretic analysis,¹ the composition

¹ Electrophoretic analysis was performed in a diethylbarbiturate buffer of pH = 8.6 with ionic strength of 0.1 according to the technique of Longworth, L. G., *Chem. Rev.*, 1942, 30, 323.

of the four samples of casein used. These results indicate that the commercially available preparations of casein as well as the casein obtained by the acidification of skim milk have a fairly constant composition consisting of 79 to 81 per cent of the fast moving component and of 19 to 21 per cent of the slow moving component.

TABLE III

Determination of a Standard Enzyme Solution Using Different Preparations of Casein

Casein used	Solution used	G*	Activity found	Composition	
				Fast component	Slow component
	<i>per cent</i>			<i>per cent</i>	<i>per cent</i>
K.B. casein†.....	0.25	210	990	79	21
Merck casein§.....	0.25	208	1000	81	19
Borden 453 	0.26	208	950	81	19
Freshly precipitated casein¶.....	0.24	210	935	81	19

* G = galvanometer reading of the precipitate obtained by the addition of 3.0 ml. of 5 per cent trichloroacetic acid to 0.50 ml. of the casein solution and 1.50 ml. of saline.

† Obtained from California Milk Products Company.

§ Obtained from Merck and Company.

|| Obtained from The Borden Company.

¶ Precipitated by adjusting the pH of skim milk to 4.4.

RESULTS

(a) *Determination of Activities of a Number of Proteolytic Enzymes of Different Origin.*

We have determined the activities of two crystalline and one purified enzymes from beef pancreas and also enzymes of bacterial and fungal origins (Table IV). The crystalline chymotrypsin was found to have the highest activity per milligram N, being approximately two and one-half times more active than crystalline trypsin, according to this test. However, both crystalline enzymes are more active than any of the commercial proteinases isolated from pancreases, bacteria, or fungi.

(b) *The Rate of Activation of Acid Extract of Beef Pancreas.*

The measure of activity has also been used to determine the rate of activation of an acid extract of beef pancreas at different pH. One hundred and fifty gm. of freshly ground beef pancreas was extracted in a Waring blender for 5 minutes with 290 ml. of water and 12.5 ml. of 6 N H₂SO₄. The pH of the suspension was raised to about 4.2 with 10 ml. of 6 N NaOH and filtered. The pH of aliquot samples of the filtrate was adjusted to a range between

4.2 to 8.9 and kept in ice water mixture. Aliquot samples were taken for activity determination at different time intervals. The results of such an experiment (Table V) demonstrate that the enzyme solution is activated at least 50-fold at pH between 7.7 and 8.2, but not at pH 4.2. Other experiments with homogenized hog and bovine pancreas indicate that the activation of the enzymes likewise takes place more rapidly at the higher pH and

TABLE IV

Comparison of Proteolytic Activities of Enzymes Obtained from Different Sources (Digestion of Casein at pH 7.6)

Origin	Enzyme	Units/mg. N
Beef pancreas	Crystalline trypsin	700
Beef pancreas	Crystalline chymotrypsin	1900
Beef pancreas	Wilson's trypsin	132
Fungus	Orthozyme 10-F	345
Fungus	Fungal enzyme	30
Bacteria	Protease 15-F 2.5	338
Bacteria	Bacterial proteinase	210

TABLE V

Rate of Activation of an Acid Extract of Beef Pancreas at 0°C.

Initial pH.....	8.88	8.24	7.70	6.25	4.19
hrs.	Units/ml.	Units/ml.	Units/ml.	Units/ml.	Units/ml.
0	14	—	17	—	15
6½	18	16	18	—	—
22½	100	145	71	20	—
46½	610	515	580	288	18
71	685	760	715	327	14
Final pH.....	8.02	7.45	7.28	6.30	4.17

that by this type of estimation pancreas from each of these two species has approximately the same unit activity per gram of glands, namely, 2600 for hog and 2800 for bovine.

SUMMARY

A method has been described for the determination of proteolytic activities of enzyme preparations using casein as substrate. The rate of digestion is proportional to the enzyme concentration used. This relationship is utilized as a measure of the enzyme activity. One unit of activity is defined as the amount which is required to digest casein in 15 minutes at 37.5°C. so that 50 per cent of the protein in 1 ml. of 0.25 per cent solution is not precipitable by

trichloroacetic acid. This method has been used to determine the activity of enzymes from different sources and also used to follow the rate of activation of enzymes.

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ISOLATION OF CRYSTALLINE RICIN*

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1. INTRODUCTION

A crystalline material possessing powerful toxicity has been isolated from crude extracts of castor bean meal. The crystalline material is a protein of the globulin type.¹ It is soluble in acid or alkaline solution and is least soluble in the range of pH 5.0 to 8.0. The ultraviolet light absorption spectrum of the crystalline protein is similar to that of a typical protein. The toxicity of the isolated crystals is higher than that of the mother liquor freed of the crystals. On repeated recrystallization the toxicity of the mother liquor approaches that of the crystals. Ultracentrifuge and electrophoresis measurements of a sample of three times recrystallized material showed that the material is fairly homogeneous. Solubility measurements, however, indicate that the crystalline protein even after several recrystallizations apparently consists of a solid solution of more than one component. The method of separation of the crystalline protein into its possible components is still unavailable.

2. Method of Isolation of Crystalline Ricin

The crystallization of ricin from crude extracts of castor bean meal proceeds best in the presence of sodium sulfate or ammonium sulfate.

(a) *Crystallization in the Presence of Sodium Sulfate.*—A solution of crude Na_2SO_4 -ricin² in water yields crystals of toxic protein when stored for several weeks at about 5°C. The details are as follows:—

10 gm. of dry powder of Na_2SO_4 -ricin is suspended in 30 to 40 ml. water. It is best to add the powder slowly to the measured amount of water so as to allow the powder to become wet gradually. The mixture is stirred until uniformly dispersed. It is then filtered clear on fluted paper. The filtration, if slow, is allowed to proceed overnight in the cold room at about 5°C. The solu-

* This paper is based on work done for the Office of Scientific Research and Development under Contract OEMsr-129 with The Rockefeller Institute for Medical Research.

The experiments referred to were first reported July 15, 1944.

¹ The isolation of a non-toxic crystalline globulin from castor bean has been reported by Ritthausen in 1881 (1) and by Osborne in 1892 (2).

² This preparation is an aqueous extract of castor bean meal, precipitated by saturation with sodium sulfate (3). The precipitate is filtered off and dried.

tion is adjusted with 1 M sodium hydroxide or hydrochloric acid to pH 6.8³ and stored at 5°C. A slight precipitate of rosettes of very fine needles generally appears within 10 days (or longer). The bulk of the precipitate increases gradually until it reaches a maximum in 6 to 8 weeks. Occasional stirring accelerates the rate of formation of crystals, which is also greatly increased on inoculation with a large amount of crystals. The crystals are filtered or centrifuged. The yield is about 0.5 gm.

Recrystallization.—The crystalline precipitate is suspended in a volume of water equal to about one-fourth of the volume of water used for the first crystallization. Enough 1 M hydrochloric acid is added slowly with stirring until the crystals dissolve. The solution is filtered clear on fluted paper and the paper is washed with a small amount of 0.01 M hydrochloric acid. The filtrate and washings are titrated to pH 6.8 with 1 M sodium hydroxide, but the addition of sodium hydroxide is interrupted at the first appearance of turbidity even if pH 6.8 is not reached. The solution is stored at 5°C. A heavy crop of crystals generally is formed within 24 hours and the crystallization is complete in 2 or 3 days.

(b) *Crystallization in the Presence of Ammonium Sulfate.*—10 gm. dry Na_2SO_4 -ricin powder is stirred up with 30 ml. of water. The mixture is filtered on fluted paper; the residue on the paper is washed with about 10 ml. of water. Enough solid ammonium sulfate is added to the combined filtrate and washings so as to bring the solution to 0.8 saturation (5.6 gm. per 10 ml.). The precipitate formed is filtered with suction. The filter cake is weighed and then dissolved in water in proportion of 1.3 ml. of water to 1 gm. of filter cake. Saturated ammonium sulfate is added slowly until a slight turbidity appears which is removed by filtration through folded paper. The filtrate is titrated to pH 6.8 with 1 M sodium hydroxide. The solution is left for 2 to 3 days at 5°C. A heavy precipitate gradually forms. The suspension is centrifuged at a temperature not higher than 10°C. The residue is dissolved in about 3 ml. of water and stored at 5°C. A heavy amorphous precipitate forms in a few hours. The amorphous precipitate gradually changes into fine needles. The suspension is filtered or centrifuged after 2 or 3 weeks.

The method for recrystallization is the same as described in section (a).

The mother liquors from the various crystallizations generally yield more crystalline protein when brought to 0.8 saturation with solid ammonium sulfate. The precipitate formed is dissolved in about an equal weight of water and stored at 5°C. A heavy crop of crystals is formed within 2 or 3 weeks.

3. Form of Crystals

The material generally crystallizes in the form of rosettes of fine needles (Fig. 1 a). Large prismoidal crystals appear if recrystallization takes place slowly from dilute solution (Fig. 1 b).

³ The pH is checked by the drop method on a test plate.

4. Toxicity Tests

The toxicity of the first crystals was tested by Professor A. H. Corwin. 1 mg. of the crystalline protein was found to have a toxicity equivalent to 2 mg.

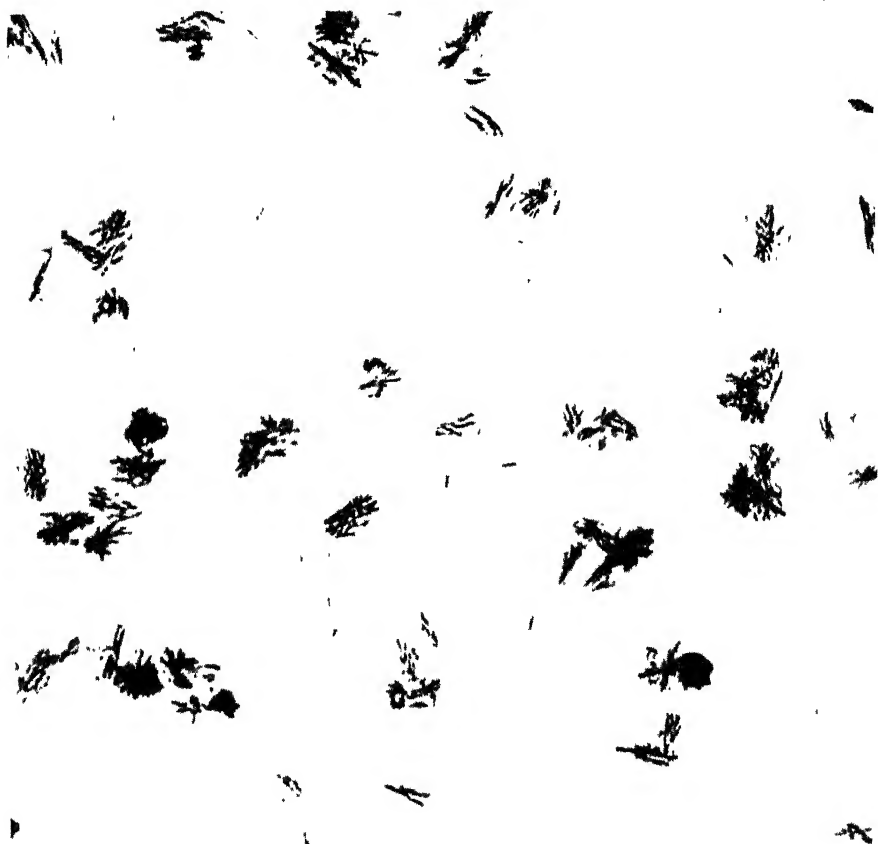


FIG. 1a

FIGS. 1a and 1b. Crystals of ricin.

of protein of the crude Na_2SO_4 ricin, whereas the toxicity of 1 mg. of protein in the mother liquor of the same crystals was equivalent to only 1.3 mg. protein of Na_2SO_4 ricin. This indicated a sharp fractionation in favor of the crystals and is evidence that the toxicity is really a property of the crystalline protein.

The toxicity of three times recrystallized ricin protein was measured in Dr. R. Keith Cannon's laboratory. The toxicity of the crystals was 680 T.U. per mg. protein while the toxicity of the mother liquor was 520 T.U. per mg. protein.

The specific toxicity of five times recrystallized protein and of the mother liquor was determined in Dr. R. Keith Cannan's laboratory. No significant difference in the specific toxicity of the crystals and the mother liquor was found. This result shows that the present method of recrystallization no longer

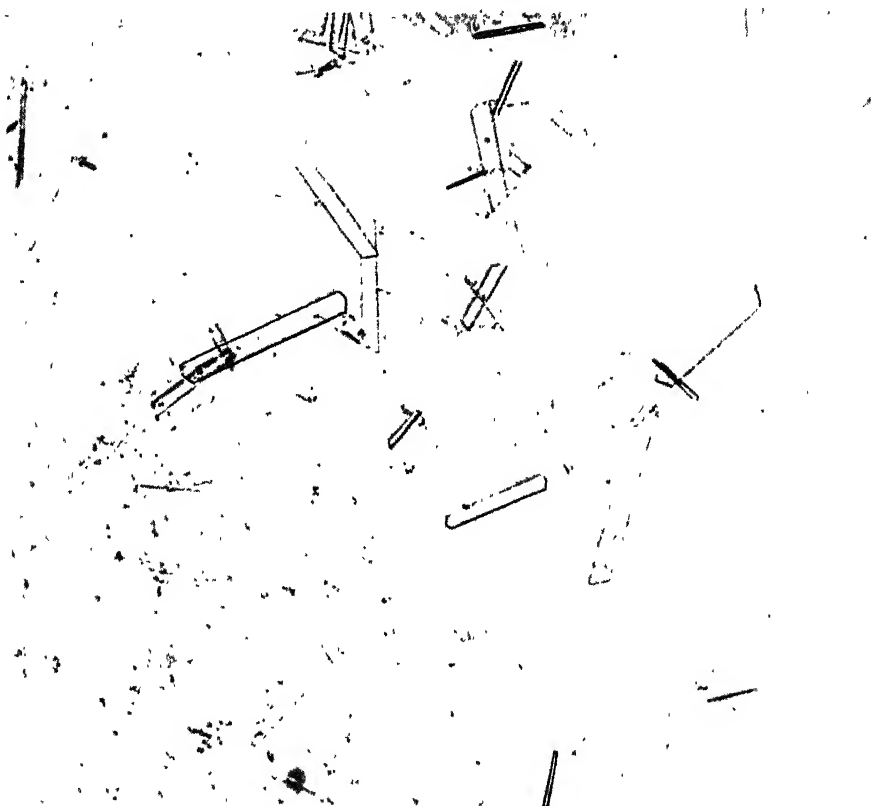


FIG. 1 b

changes the composition of the crystalline protein sufficiently to be detected by differences in toxicity.

5. Some of the Protein Properties of Crystalline Ricin¹

Crystalline ricin appears to be a protein of the globulin type, with an isoelectric point reported (3) to be at pH 5.4 to 5.5. It is least soluble in the range of pH 5.0 to 8.0. It is precipitated in 0.15 M trichloroacetic acid. Its ultra-

¹ Additional information on the properties of crystalline ricin is to be found in the publication of Kabat, Heidelberger, and Bezer (3).

violet absorption spectrum (Fig. 2) resembles that of other proteins with a maximum absorption at a wave length at 279 $m\mu$ and a minimum at 250 $m\mu$.

6. Purity of Three Times Crystallized Ricin

(a) *Ultracentrifuge Method* (Dr. M. A. Lauffer).—Solution used: 1.5 per cent solution of three times crystallized ricin in 0.2 M sodium chloride made up in 0.05 M acetic acid; final pH about 3.5. The solution was filtered clear on No.

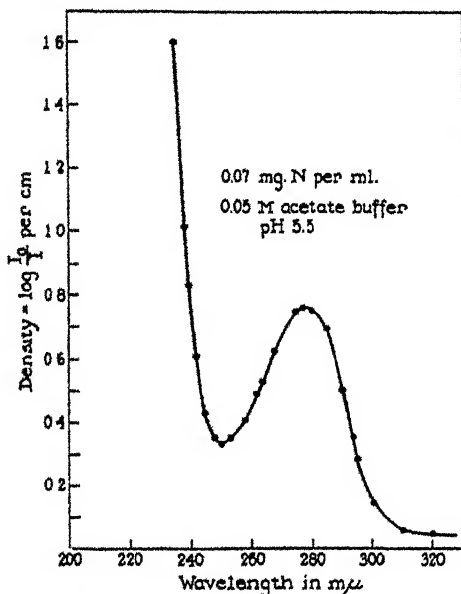


Fig. 2. Ultraviolet absorption spectrum of crystalline ricin.

42 Whatman paper. The data were recorded by the Svenson schlieren method at 10 minute intervals. Only one moving boundary was observed throughout the 2 hours' centrifugation and the symmetry of the schlieren curves indicated fair homogeneity of the material.

Sedimentation constant corrected to water at 20°C. = 3.9×10^{-13} cm./sec./unit field. Molecular weight = 36,000 (assuming the particles to be spheres with a specific volume of 0.73).

(b) *Electrophoresis* (Dr. M. A. Lauffer).—Solution used: 0.63 per cent solution of three times crystallized ricin in 0.02 M sodium chloride made up in 0.05 M acetic acid. The same electrolyte solution was used to fill the upper compartments of the Tiselius apparatus. Current passed at the rate of 10 milliamperes for 1.5 hours. Boundary recorded at the end of the experiment by the Longworth schlieren scanning method. The electrophoretic pattern indicated the presence of only one moving component.

(c) *Solubility Test.*—Measurements were made of the solubility in 0.05 M acetate buffer pH 5.5 of four times recrystallized ricin in the presence of increasing amounts of crystals of ricin in suspension.

Experimental Procedure.—5 gm. of three times recrystallized filter cake were dissolved in 15 ml. 0.1 M acetic acid and filtered clear. The solution was brought to pH 5.5 by means of 15 ml. 0.1 M sodium hydroxide and left for several days at 5°C. for crystallization. The suspension of crystals was filtered on Whatman's No. 42 paper at about 20°C. The crystals were then washed twice by resuspending in 30 ml. 0.05 M acetate buffer pH 5.5 and refiltering on No. 42 paper.

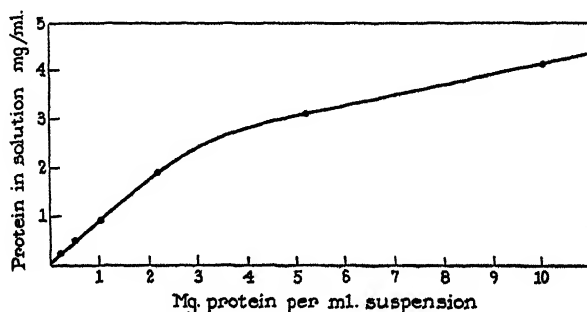


FIG. 3. Solubility of three times crystallized ricin in presence of increasing quantities of solid phase.

The concentration of protein in the filtrates was found to be as follows:

Mother liquor.....	3.0 mg./ml
First washing.....	3.1 mg./ml
Second washing.....	3.3 mg./ml

The washed crystals were resuspended in 30 ml. 0.05 M acetate buffer pH 5.5. Increasing amounts of the concentrated suspension of crystals were then pipetted into 15 ml. test tubes each provided with a pyrex glass bead. The tubes were nearly filled with the same buffer solution, stoppered with one-hole rubber stoppers, and then plugged with short glass rods. Care was taken not to leave any air bubbles in the tubes. The suspensions were revolved mechanically with a slow motion for 18 hours at about 20°C., then filtered on small No. 42 filter papers. The filtrates, as well as the suspensions before filtration, were analyzed for protein by the copper-phenol method of Herriott (4). The data are given graphically in Fig. 3. The experiment shows that the solubility of the crystals of ricin is not independent of the total amount of the excess crystals in suspension, but continues to increase gradually in the presence of

increasing amounts of the solid phase. The curve⁵ resembles the theoretical solubility curve of a solid solution of two or more components (5). Since more than one component is present it is possible that the agglutinating properties of the preparation are due to one protein and the toxic properties to another protein.

7. SUMMARY

A toxic crystalline protein has been isolated from crude extracts of castor bean meal. Ultracentrifuge and electrophoresis tests show the crystalline protein to become fairly homogeneous after three or four crystallizations. This is also confirmed by toxicity measurements. Solubility tests, however, indicate the presence of more than one protein component in the crystalline material, possibly in the form of a solid solution which cannot be separated into its components by repeated crystallization under the present technique.

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⁵ Such curves are frequently found with proteins which appear to be homogeneous by ultracentrifuge or electrophoresis methods. The constant solubility test is much more sensitive since it will detect the presence of a mixture or solid solution of proteins even though the various components have the same solubility. The electrophoresis or ultracentrifuge technique, on the other hand, can detect only proteins which have different rates of sedimentation or electrophoresis.

PROTEOLYTIC CONTAMINANTS OF CRYSTALLINE RIBONUCLEASE

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Several workers (1-5) have reported that crystalline ribonuclease (6) can hydrolyze proteins, but they have not demonstrated whether this was due to an intrinsic property of the ribonuclease molecule or to the presence of impurities. Schneider (7) showed that the inhibitory effect of ribonuclease on succinic dehydrogenase was apparently due to a contaminant (possibly a proteolytic enzyme) in the crystalline ribonuclease. Kunitz (6) noted the possible presence of a small amount of impurities in the sample of ribonuclease used for solubility tests. Experiments in this laboratory have confirmed the presence of proteolytic activity in every sample of crystalline ribonuclease tested and have shown that all of this activity was due to impurities.

EXPERIMENTAL

Samples of crystalline ribonuclease prepared by the method of Kunitz (6) were tested for their ability to digest denatured hemoglobin (8). Several representative assays are given in Table I. Every specimen checked showed some protease activity, and many of the samples also manifested additional protease activity after activation with trypsin, thereby indicating the presence of proteolytic precursors. The total quantity of protease activity and the ratio of protease to nuclease activity, however, varied enormously with the different preparations, showing that at least part of the proteolytic activity was due to impurities. This was confirmed by experiments on the differential destruction of the various activities of the ribonuclease preparations. The effect of pH and of salt concentration on the heat inactivation of ribonuclease and its concurrent protease activity is shown in Tables II and III. The data show that the ratio of nuclease to protease activity was not constant and that under very specific conditions it was possible to destroy all the protease activity while leaving the nuclease activity practically intact. Protease activity is therefore not an inherent property of the ribonuclease molecule.

The proteolytic activity of the samples of ribonuclease tested could have been due to contamination by both chymotrypsin and trypsin, since they were found to clot milk (9) and to hydrolyze benzoyl-L-arginineamide¹ (10). No tests were made for other peptidases. Assuming that one-half of the proteolytic activity was due to trypsin and one-half to chymotrypsin, the median

¹ The author is greatly indebted to Dr. Joseph S. Fruton of the Yale University School of Medicine for the preparation of benzoyl-L-arginineamide used in these tests.

TABLE I
Assay of Various Samples of Crystalline Ribonuclease

Sample	Ribonuclease activity	Proteolytic activity	Potential proteolytic activity
	[N.U.] per mg. N	10^{-6} [T.U.] ^{Hb} per mg. N*	
Ribonuclease 1	1115	20	<50
" 2	1080	38	<50
" 3‡	1050	50	<50
" 4‡	1040	26	<50
" 5	1025	65	<50
" 6	1010	340	13,000
" 7	1010	25	<50
" 8‡	1000	1824	816
" 9	996	1190	2,460
" 10	890	702	20,000

* 1 mg. trypsin N is equivalent to $150,000 \times 10^{-6}$ [T.U.]^{Hb}; 1 mg. chymotrypsin N is equivalent to $40,000 \times 10^{-6}$ [T.U.]^{Hb}.

‡ The author is indebted to Dr. M. Kunitz of The Rockefeller Institute for Medical Research for these three samples of crystalline ribonuclease.

TABLE II
The Effect of pH on the Differential Inactivation of Nuclease and Protease Activities of Crystalline Ribonuclease

Experimental Procedure.—A series of tubes, each containing 5 ml. of 0.1 per cent ribonuclease in various solvents, was placed in boiling water. Aliquots were removed at various time intervals, cooled rapidly in ice water, allowed to stand at 5°C. for 1 hour, then adjusted to (a) pH 4.0 for assay of ribonuclease activity or (b) pH 1.0 for assay of proteolytic activity.

Solvent.....	0.1 N hydrochloric acid	0.1 N acetic acid	0.05 M acetate buffer	0.05 M veronal buffer	0.05 M veronal buffer	0.05 M veronal buffer
pH of solution (glass electrode)	1.1	3.1	5.5	6.8	7.5	8.0
Length of time at 100°C., min.	Ribonuclease activity left, per cent					
1	90	93	79	79		
5	54	83	71	72	68	33
15	37	77	69	60	19	0
	Proteolytic activity left, per cent					
1	71	80	48	29		
5	44	57	42	24	13	3
15	19	38	21	16	2	1

* These results are not in agreement with those of Baker and Sanders (12) and Sanders (13), who state, without giving any experimental data, that the proteolytic activity of ribonuclease solutions is destroyed at 80–100°C. at slightly alkaline pH, the ribonuclease activity being unaffected.

value found for the proteolytic impurities was 0.07 per cent, the range varying from 0.02 to 2 per cent. Even these small amounts of contaminants, however, may lead to erroneous conclusions when ribonuclease is used as a specific tool. At least one example of this has already been published (11, 7). The increase in ability to digest denatured hemoglobin noted in some samples of ribonuclease

TABLE III

The Effect of Salt on the Differential Inactivation of Nuclease and Protease Activities of Crystalline Ribonuclease

Experimental Procedure.—Tubes, containing 5 ml. of 1.25 per cent solutions of ribonuclease in various concentrations of ammonium sulfate and adjusted with 1 N sulfuric acid to pH 3, were placed in boiling water for 5 minutes, cooled rapidly in ice water to 5°C., and left at 0–5°C. for 1 hour. Aliquots were then analyzed for ribonuclease, proteolytic, and potential proteolytic activity.

Final concentration of ammonium sulfate	Ribonuclease activity	Proteolytic activity	Potential proteolytic activity
Saturation	Per cent remaining after 5 min. at 100°C.		
0	86	74	94
0.05	88	29	0
0.10	94	12	0
0.15	91	4	0
0.20	92	2*	0
0.25	90	6	0
0.30	97	9	0

* This residual proteolytic activity is in the precipitate which forms during the heating process. No proteolytic, but all of the ribonuclease, activity is present in the filtrates from such suspensions (14).

after activation with trypsin was not due primarily to chymotrypsinogen, since there was little concurrent increase in milk-clotting ability. Calculated as trypsinogen, this impurity constituted from 0 to 12 per cent of the total protein of the ribonuclease preparations assayed.

DISCUSSION

Ribonuclease is known to be thermostable over a wide range of pH (6). It has therefore been more or less assumed that, if solutions of ribonuclease are boiled, all contaminants are destroyed but the ribonuclease itself is not affected. Chantrenne (15), for example, has stated, without giving experimental data, that boiling solutions of ribonuclease for 3 minutes assures the destruction of traces of enzymes which might contaminate the ribonuclease, without affecting the activity of the latter, which is remarkably thermostable. Sanders (13) has stated similarly that since it has been suggested that even crystalline ribonuclease has some residual proteolytic activity, owing presumably to adsorbed impurity, this is destroyed by heating the ribonuclease solution to 80°C. for 10 minutes. As noted by Dubos (16), however, other enzymes (such as lysozyme) may possess physicochemical properties similar to those of ribonuclease.

Trypsin, too, is thermostable under certain conditions (17). The data of Tables II and III show that the prerequisites for the complete heat destruction of proteolytic contaminants without destruction of the ribonuclease molecule are very specific. Statements such as those of Chantrenne and Sanders would therefore appear to be meaningless, unless the exact conditions (concentration of enzyme, concentration of salt, pH, etc.) under which the heating is done are specified, and the amounts of ribonuclease and its contaminants actually determined.

The difficulties of establishing the purity of enzyme (or other protein) preparations are well known. They may be homogeneous in the electrophoresis apparatus and in the ultracentrifuge, and adhere to the phase rule requirements for the solubility of a pure substance, and still contain minute quantities of impurities. Crystallinity, whether of simple inorganic salts or complex proteins, is certainly not in itself evidence of purity. The data of Table I show that different samples of crystalline ribonuclease, prepared by identical procedures, vary enormously in the amounts of contaminants they contain. In view of these findings, the dangers inherent in the current widespread and rather indiscriminate use of enzymes as specific tools, without assaying them for all possible interfering impurities, cannot be too strongly emphasized. Had Potter and Albaum (11), to give just one example, tested their ribonuclease preparation for proteolytic activity, they would probably not have made the unqualified statement that ribonuclease *per se* inhibited Co I-cytochrome *c* reductase, succinic dehydrogenase, and cytochrome oxidase.

Methods

1. *Nitrogen*.—Assayed by Herriott's colorimetric procedure (18); checked in some cases by micro-Kjeldahl.

2. *Ribonuclease Activity*.—Determined by Kunitz's uranium-acetate procedure (6).

3. *Proteolytic Activity*.—Many samples of ribonuclease were found to inhibit the action of trypsin, and to a lesser extent that of chymotrypsin, at pH 7 but not at pH 1. It is therefore impossible to determine quantitatively the actual amount of proteolytic activity present in any sample of ribonuclease by merely adding an aliquot of a concentrated aqueous solution to a protein substrate. The following procedure has given reproducible results, checked by adding known amounts of trypsin and chymotrypsin to a solution of "proteolytic-free" ribonuclease.

Solutions of ribonuclease in 0.1 N hydrochloric acid containing 0.04 mg. nitrogen per ml. are left at 0–5°C. for 1 hour; then 1 ml. aliquots (without neutralization) are assayed as described by Anson for the estimation of trypsin (8), digestion being allowed to proceed for 5, 24, and 72 hours instead of 10 minutes.

4. *Potential Proteolytic Activity*.—When trypsin was added to some samples of ribonuclease it was noted that the proteolytic activity gradually increased owing to proteolytic precursors present as impurities in these samples. The following procedure has been used to give a qualitative assay of the amount of zymogen present.

Mixture of 4 ml. of an aqueous solution of ribonuclease containing approximately 1

mg. nitrogen per ml., plus 5 ml. 0.2 M phosphate buffer pH 7.6, plus 1 ml. of a solution of trypsin, in 0.0025 M hydrochloric acid, containing approximately 5×10^{-4} [T.U.]¹⁶ per ml., are kept at 0–5°C. Aliquots are removed daily, brought to pH 1 by the addition of an equal volume of 0.3 N hydrochloric acid, further diluted as necessary with 0.1 N hydrochloric acid, left at 0–5°C. for 1 hour, then assayed for trypsin by Anson's hemoglobin method (8). The difference in activity between the sample at zero time and after complete activation (usually 7 days) is taken as the measure of the amount of proteolytic precursor present.

The author was assisted in this work by Miss Ruth Wyman; her contributions are gratefully acknowledged.

SUMMARY

1. The ability of crystalline ribonuclease to hydrolyze proteins is due to impurities present in the preparations and not to an intrinsic property of the ribonuclease molecule. The amounts of these impurities vary enormously with different preparations.

2. The dangers inherent in the use of crystalline enzymes as specific tools, without assaying them for all possible interfering impurities, are emphasized.

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A METHOD FOR THE PREPARATION OF "PROTEASE-FREE" CRYSTALLINE RIBONUCLEASE

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A method for the preparation of crystalline ribonuclease was described by Kunitz (1) in 1940. Since that time various workers (2-6) have shown that at least some samples of ribonuclease so prepared had, in addition to the ability to degrade ribonucleic acid, the ability to hydrolyze proteins. That the latter was due to contaminants and not to an intrinsic property of the ribonuclease molecule has already been demonstrated (7, 8). The present paper describes a method for the preparation of crystalline ribonuclease free from all measurable traces of proteolytic enzymes.

Although the method for the preparation of crystalline ribonuclease described below differs essentially from that of Kunitz in only two major respects, several minor changes have also been made. It therefore seems most useful to describe the complete procedure as it is now used in this laboratory. The first essential change consists of an additional step in the preliminary treatment; namely, boiling the crude ribonuclease preparation in 0.2 saturated ammonium sulfate at pH 3. This destroys all proteolytic and potential proteolytic activity but leaves the ribonuclease molecule practically intact. The second essential change is in the pH of the solution from which the crystals separate. Crystallization is most consistent, and the yields largest, when the pH of this solution is 4.6.

The saturated ammonium sulfate is prepared at 20-25°C. (760 gm. of salt per 1000 ml. distilled water). Determinations of pH are made on a test plate by mixing 1 drop of the appropriate 0.01 per cent indicator with 1 drop of the solution to be tested, and comparing the color with that formed by 1 drop of the same indicator with 1 drop of 0.1 M standard buffer of the desired pH.

The extent of purification of the ribonuclease in an average preparation is shown in Table I. Twelve such preparations have now been made and assayed. All were tested for their ability to clot milk and to hydrolyze denatured hemoglobin, egg albumin, protamine (salmon), histone (calf thymus), and benzoyl-L-arginineamide. In no case was any trace of protease activity detected. Acidified solutions of these preparations containing 0.04 mg. of nitrogen per ml. when left in contact with denatured hemoglobin (8) for 96 hours at 25°C. gave no increase in split products not precipitable with trichloroacetic acid. They therefore contained less than 0.002 per cent of proteolytic contaminants (calculated

as trypsin). The effect of two of these preparations on succinoxidase has been determined by Dr. Walter C. Schneider of the University of Wisconsin by methods previously described (7). Both samples inhibited slightly the succinoxidase activity of mouse liver homogenates; the mechanism of the inhibition has not been established.

TABLE I
Extent of Purification of Ribonuclease

Preparation	Ribonuclease activity		Proteolytic activity*	Potential proteolytic activity*
	Total [N.U.]	[N.U.] ^a per mg. N	10 ⁻⁴ [T.U.] ^b	per mg. N
Preliminary purification				
Acid extract from				
12.5 liters ground pancreas.....	2,000,000	33		
Fraction A.....	1,200,000	508	900	7,000
Fraction B.....	984,000	718	Trace	Trace
Fraction C.....	886,000	740	<20	<50
Crystallization from ammonium sulfate				
First crystals.....	585,000	836	<20	<50
First mother liquor.....	289,000	570		
Second crystals.....	471,500	936		
Second mother liquor.....	113,000	719		
Third crystals.....	315,900	1030		
Third mother liquor.....	135,300	806		
Fourth crystals.....	233,450	1040		
Fourth mother liquor.....	110,500	1060		
Crystallization from alcohol†				
after fourth crystallization from ammonium sulfate				
Dialyzed ribonuclease.....	198,450	1037		
First crystals.....	188,500	1031	<1	<50

* Assayed as described previously (8).

† By the method of Kunitz (1).

1. *Preliminary Purification and Concentration.*—Remove pancreas¹ from cattle immediately after slaughter and immerse in ice cold 0.25 N sulfuric acid. The pancreas can either be stored in this solution at 0-5°C. for several days, or used immediately. Remove fat and connective tissue, and mince in meat grinder. Suspend each liter² of ground pancreas in 2 liters of ice cold 0.25 N sulfuric acid, and allow suspension to stand at 0-5°C. for 18 to 24 hours with occasional stirring. Strain the suspension through fine cheese-cloth, resuspend the residue in an equal volume of cold

¹ Frozen pancreas, obtainable from any of the large slaughter-houses, can be used if only ribonuclease is to be prepared.

² This expression is used to denote the relative amounts of material used. It does not mean that each liter or gram of material is processed separately.

0.25 N sulfuric acid, and re-strain after 1 hour. Reject residue. Add 430 gm. of ammonium sulfate to each liter of combined filtrates; final concentration equals 0.65 saturation of ammonium sulfate. Filter through fluted paper (Eaton and Dikeman No. 612 or Whatman No. 2). Save filtrate. Suspend precipitate in a volume of cold water equal to that of the original minced pancreas. Add 430 gm. of ammonium sulfate per liter suspension; refilter through fluted paper. Reject residue or save for the isolation of chymotrypsinogen, etc., as described by Kunitz and Northrop (9). Combine filtrates and bring to 0.8 saturation of ammonium sulfate by adding 105 gm. of salt per liter. Filter with suction on large Büchner funnel, using hardened paper (Schleicher and Schüll No. 575). Reject filtrate. Filter cake = "fraction A;" yield is about 4 gm. per liter of ground pancreas used. Store at 0 to 5°C. until approximately 50 gm. are collected.

2. *Removal of Proteolytic and Potential Proteolytic Activity.*—Dissolve each gram² of fraction A in 5 ml. distilled water and pour into 20 ml. of 0.2 saturated ammonium sulfate previously adjusted with sulfuric acid to pH 3 and heated to 95–100°C. Stir 5 minutes at 95–100°C., then cool quickly to 25°C. Leave at 20–25°C. for 1 hour. Add 1 gm. of Standard super-cel per 100 ml. suspension, filter with suction through soft paper (Eaton and Dikeman No. 303), and wash filter cake three times with small quantities of 0.2 saturated ammonium sulfate. Reject residue. Dissolve 18.8 gm. of ammonium sulfate in each 100 ml. of filtrate; final concentration equals 0.5 saturation. Add 1 gm. Standard super-cel per 100 ml. suspension and refilter with suction through soft paper. Reject residue. Dissolve 21 gm. of ammonium sulfate in each 100 ml. filtrate; final concentration equals 0.8 saturation. Filter with suction on hardened paper. Reject filtrate. Filter cake = "fraction B;" yield is approximately 0.7 gm. per gm. fraction A used.

Dissolve each gram of fraction B in 5 ml. distilled water, adjust solution to pH 4.8 with a few drops of 5 N sodium hydroxide, then add 5 ml. saturated ammonium sulfate. Filter with suction through soft paper with the aid of 1 gm. Standard super-cel per 100 ml. suspension. Discard residue. Adjust filtrate to pH 4.2 by means of 1 N sulfuric acid and then add slowly, stirring constantly, 67 ml. of saturated ammonium sulfate per 100 ml. filtrate; final concentration equals 0.7 saturation. Filter with suction on hardened paper. Discard filtrate.³ Filter cake = "fraction C;" yield is about 0.7 gm. per gm. fraction B used.

3. *Crystallization.*—Dissolve each gram of fraction C in 1 ml. distilled water. Filter with suction through soft paper with the aid of 5 gm. filter-cel per 100 ml. solution and wash residue several times with small quantities of water. Reject residue. Dilute combined filtrate and washings with water to 2 ml. Add saturated ammonium sulfate *slowly* with stirring until a very faint turbidity appears (about 40 ml. per 100 ml. solution) and immediately adjust the pH of the mixture to 4.6. The solution clears rapidly. Leave at 20–25°C. for 3 days. Crystals of ribonuclease gradually form.⁴ Filter with suction on hardened paper. Filter cake = first ribo-

³ The yield of ribonuclease can be increased by adding ammonium sulfate to this filtrate to 0.8 saturation, filtering on hardened paper, and reworking the filter cake with the next batch of fraction B.

⁴ If too much ammonium sulfate has been added, an amorphous precipitate will form rapidly. This may change within 1 or 2 days into a mass of fine crystals; if not,

nuclease crystals; yield is about 0.6 gm. per gm. fraction C used. Adjust filtrate to pH 4.2; add saturated ammonium sulfate slowly, with stirring, to 0.8 saturation. Filter with suction on hardened paper. Discard filtrate. Filter cake = first ribonuclease mother liquor;⁶ yield is about 0.2 gm. per gm. fraction C used.

4. *Recrystallization*.—Dissolve each gram of ribonuclease crystals in 2 ml. water. Filter with suction through soft paper with the aid of 5 gm. filter-cel per 100 ml. solution. Wash residue several times with small amounts of water, and dilute combined filtrate and washings to 3 ml. Add saturated ammonium sulfate *slowly*, with stirring, to very faint turbidity (about 40 ml. per 100 ml. solution). Leave at 20–25°C.; crystals form rapidly. Filter with suction on hardened paper after 2 days. Filter cake = ribonuclease crystals; yield is about 0.6 gm. per gm. of crystalline filter cake used. Adjust filtrate to pH 4.2. Add saturated ammonium sulfate slowly, to 0.8 saturation. Filter with suction on hardened paper. Discard filtrate. Filter cake = ribonuclease mother liquor;⁶ yield is about 0.3 gm. per gm. of crystalline filter cake used.

The author was assisted in this work by Miss Ruth Wyman whose contributions are here gratefully acknowledged. The author also wishes to thank Dr. Walter C. Schneider for his kindness in determining the effect of the ribonuclease preparations on succinoxidase.

SUMMARY

A method is described for the preparation of crystalline ribonuclease free from all measurable traces of proteolytic enzymes.

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the solution should be adjusted to pH. 4.2, brought to 0.8 saturation with saturated ammonium sulfate, filtered with suction on hardened paper, and the filter cake processed like fraction C.

⁶ Additional ribonuclease crystals can be obtained by reworking these filter cakes like fraction B.

THE CHANGE IN OSMOTICALLY INACTIVE FRACTION PRODUCED BY CELL ACTIVATION*

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I

The Osmotically Inactive Substance

The study of cells approximately spherical in configuration, represented most closely by eggs of marine invertebrates, has led to the well established result (summarized by Lucké and McCutcheon (1932)) that these cells, as well as others (e.g., erythrocytes (Hamburger (1898)) and leucocytes (Shapiro and Parpart (1937)) obey within limits the law of Boyle-van't-Hoff, so that the total cell volume varies inversely with the "activity" of the external solution with which it is in equilibrium osmotically. Stated symbolically,

$$PV = K \quad (1)$$

where P and V are the pressure and volume, and K is a constant. The cells which have been studied do not obey this simple relation unless a correction factor b , termed variously the "non-solvent space," "osmotically inactive substance," "dry substance," etc., is introduced, making the equation read

$$P(V - b) = K. \quad (2)$$

Microscopic examination of living cells reveals a variety of formed elements, which when centrifuged down can be observed to occupy a known portion of the cell volume. In the egg of the sea urchin *Arbacia punctulata*, Harvey (1932) gives the following: 1 per cent oil, 4.8 per cent mitochondria, 27.2 per cent yolk, 5.5 per cent pigment, and 0.4 per cent nucleus, totalling 38.9 per cent of the cell volume. If these formed elements took no part in the swelling and shrinking of the cell in anisotonic solutions, the value of b in Equation 2 would be at least 38.9 per cent of the cell's initial volume, but as Lucké, Larrabee, and Hartline (1935) have observed, the b value averaged 12 per cent in their measurements. The difference must reside in the fact that certain of the formed elements shrink and swell, along with the rest of the cytoplasm; for

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the nucleus this has been demonstrated to be the case (Beck and Shapiro (1936), in starfish germinal vesicle; Shapiro and Parpart (1937), in mammalian leucocytes).

The osmotically inactive fraction (o.i.f.) is of interest in that it is a quantitatively determined cell constant, and hence may afford an index to the progression of certain events within the cell. Also, in determining the "permeability constant" of the cell (a measure of the rate at which water or dissolved substances diffuse into or out of the cell (Lucké and McCutcheon (1932); Jacobs (1932))) it is essential to know the value of b to obtain the most precise values, particularly where b is large. In his calculations of the permeability constant for water in the egg of the worm *Chaetopterus pergamentaceus*, Shapiro (1941 *b*) found that the values were about 11 per cent higher when the osmotically inert material was not taken into account in the computations.

In the case of the sea urchin egg, the b value has been obtained only for unfertilized cells, and the implicit assumption made that it does not alter significantly when the cell changes from the resting to the actively dividing state. A variety of changes, chemical, physiological, and morphological, make their appearance when cellular activation (fertilization) occurs. There is an increase in oxygen uptake (Rubenstein and Gerard (1934), and many others), the dehydrogenase activity increases (Ballentine (1940)), mitotic phenomena are evident, lactic acid appears in small amount (Perlzweig and Barron (1928), Rapkine (1931), Runnström (1933), Hutchens *et al.* (1942)), echinochrome diffuses out of the cell (Shapiro (1946)), and so on. Mirsky (1936) observed that shortly after fertilization (between 3 and 10 minutes) about 12 per cent of the egg protein in *Arbacia* and in *Strongylocentrotus* coagulates and becomes insoluble. Unfertilized eggs contain 63 to 67 per cent of their dry weight as protein (Hutchens *et al.* (1942)). The net effect of these diverse changes upon the osmotically inactive fraction of activated cells has not hitherto been determined.

When the sea urchin egg is broken by centrifugal force into two viable parts known as the light and heavy half, the sum of the individual o.i.f.'s is greater than that of the parent cell. The figures arrived at by Lucké (1932) were $18,000 \mu^3$ for the whole cell, and $8,000 \mu^3$ and $15,000 \mu^3$ respectively for the light and heavy half, an increase of about 28 per cent. Owing to the higher concentration of granules in the heavy half, it is to be expected that the o.i.f. will be greater, but it is of interest that the relative granule volumes are not those indicated by these figures, again pointing to the fact that the formed elements take part in osmotic swelling. The sum of the oxygen uptakes of the unfertilized half eggs, it may be added parenthetically, is about 29 per cent greater than that of the whole unfertilized egg (Shapiro (1935)). As will appear in the sequel, the problem of the osmotically inactive substance is integral with that of volume change on activation, hence we may turn our attention briefly to the latter point.

II

Volume Change on Fertilization

The possibility of a change in volume on fertilization engaged the attention of a number of earlier workers in the field, with discordant results in one case, on the same species. Loeb (1908) found no significant change in the volume of the egg of *Strongylocentrotus* as a result of fertilization. McClendon (1910) observed a decrease in mean egg diameter from 83 to 75 when *Arbacia punctulata* eggs were fertilized. These were eggs placed in a cane sugar solution, approximately isosmotic with sea water. On the other hand, he observed on fertilization an increase in diameter from 83 to 86, of eggs in ordinary sea water. It should be pointed out here that McClendon measured only twenty eggs each for the cane sugar experiment, and ten each for eggs in ordinary sea water. In the case of the latter the diameters showed a large variation (from 75 to 90, no units stated). Glaser (1914) concluded that on fertilization the eggs both of *Arbacia* and of the starfish *Asterias forbesii* show a decrease in diameter. In discussing this with Dr. Glaser, he pointed out that his measurements were confined to the "instant" of fertilization. Okkelberg (1914) held that the eggs of the brook lamprey decrease in volume after fertilization by about 13 per cent. In his calculations the eggs were assumed to be perfect ellipsoids of revolution.

It appears from the discussions by the various authors that they expected that all eggs should show the same qualitative volume change (either increase or decrease) on fertilization, without recognizing that since they were working in some instances on different genera, the direction of change might depend upon the particular cell being studied. Some evidence for this will be presented later.

III

Technic

Eggs were obtained and handled as in earlier studies (e.g., Shapiro (1941)). The hypotonic sea waters were made up fresh from the laboratory's running sea water, which was mixed with distilled water. Owing to the variability of the o.i.f. in the eggs of different urchins, all eggs for any given series of measurements (as exemplified by the data of Fig. 1) were taken from a single female, and the values for unfertilized and fertilized eggs then compared. Two drops of egg suspension were placed in about 100 cc. of each of the hypotonic solutions, and were allowed ample time to attain equilibrium value (25 minutes or longer) at room temperature (about 25°C.). The diameters of twenty or more eggs were measured with a filar ocular micrometer at a magnification of approximately 140 diameters, the volume of each egg computed, and an average value obtained for egg volume at any given osmotic pressure. It might be noted in passing that averaging of egg diameters to obtain average egg volume, a shorter procedure, is nevertheless mathematically incorrect, since volume is not a linear, but a power function of the diameter. Lucké (1935) concluded that tempera-

ture change *per se*, when varied from 5.4° to 29.3°C., did not shift the position of osmotic equilibrium in the unfertilized *Arbacia* egg. The subsequent development of the swollen fertilized eggs, in hypotonic media, was followed after measurement. The eggs in 80 and 90 per cent sea water underwent from 85 to 100 per cent development in the different experiments; those in 70 per cent were retarded and more variable, whereas none were observed to cleave in 60 per cent sea water.

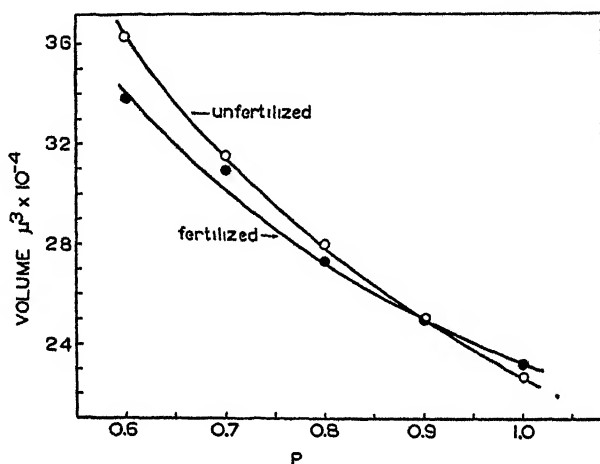


FIG. 1. Pressure-volume relationship for unfertilized and fertilized eggs of the sea urchin, *Arbacia punctulata*. The points represent equilibrium volumes of eggs swollen in various dilutions of sea water. The curves drawn in are theoretical ones calculated from $P(V - b) = K$.

IV

RESULTS

The results are summarized in Table I and in Figs. 1 and 2. K_1 and K_2 refer to relative volumes and osmotic pressures; the constants calculated in Table I are expressed in terms of the volume of the unfertilized egg as 1.000. The ordinates in Figs. 1 and 2 give the cell volumes in cubic micra. The magnitude of the o.i.f. is indicated by the departure of K_1 and K_2 from unity.

The o.i.f. shows on fertilization a large increase, varying from 136 per cent to 519 per cent. There is an average volume increase of some 2.7 per cent on fertilization, and the osmotic pressure at which the volumes of fertilized and unfertilized eggs are equal is in the region of 86 per cent sea water. Lucké *et al.* (1935) found the o.i.f. for unfertilized eggs to range in different individuals from 6 to 20 per cent; in this series it varied from 5.5 to 9.6 per cent. The variability of this cell constant in different urchins may possibly be a reflection of the variation in nutritional status of the animals. When brought into

the laboratory, they may be kept in the aquarium in running sea water for days or weeks at a time, without being fed, and yet contain at the end of these times ripe ovaries, with eggs which show normal cleavage, development, and osmotic properties.

TABLE I
Osmotically Inactive Fraction in Unfertilized and Fertilized Sea Urchin Eggs

Date	b_1 (unfertilized)	b_2 (fertilized)	$\frac{b_2}{b_1}$	Osmotic pressure where volumes are equal (calculated; sea water = 1.000)	Volume increase on fertilization	K_1	K_2
1947					per cent		
July 15.....	0.096	0.292	3.04	0.897	1.4	0.898	0.722
July 28.....	0.055	0.341	6.19	0.813	5.4	0.945	0.713
July 30.....	0.083	0.196	2.36	0.877	1.4	0.917	0.818
Average.....	0.078	0.276	3.86	0.862	2.73	0.920	0.751

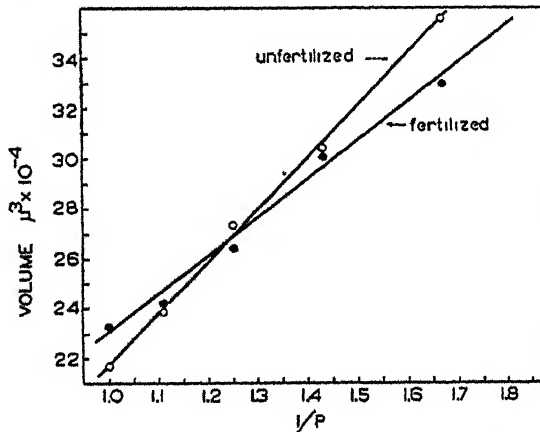


FIG. 2. Plot showing applicability of the Boyle-van't-Hoff law to another set of data. The intercept of each line at zero pressure (not shown) with the y -axis gives the osmotically inactive volume. The curve represents a close approximation to a least squares fit.

It should be pointed out that the conclusion arrived at in this paper as to the increase in cell volume of the sea urchin egg on fertilization is based not merely on the measurements in sea water alone, but is supported by the positions of the other points on the P - V plot which demonstrate that there are two distinct curves, each obeying Boyle's law, with different values for b and K .

In an earlier paper (Shapiro (1941 *b*)) b values were calculated for individual

eggs of the worm *Chaetopterus pergamentaceus*, and were found to average 38.8 per cent for unfertilized and 29.9 per cent for fertilized. This was the reverse of the result obtained in the present paper for *Arbacia*. A preliminary experiment was run on *Chaetopterus* eggs following the technic used on *Arbacia* in the present paper, viz. averaging volumes of about twenty eggs at each osmotic pressure, and a confirmatory result was obtained: b declined on fertilization in the worm eggs. Since low percentage of cleavage (30 per cent) was obtained, further work is required with these eggs in order to establish with certainty this difference between *Arbacia* and *Chaetopterus*. The qualitatively different results in the two forms are of course entirely possible, and it is of interest that such is the case. It may be pointed out moreover at this juncture that the direction of the change in b in *Chaetopterus* and *Arbacia* parallels the direction of respiratory change (oxygen uptake). *Chaetopterus* exhibits a decline in rate of oxygen uptake on fertilization (Whitaker (1933)), and *Arbacia* shows an increase (various investigators). The parallelism between the increase in

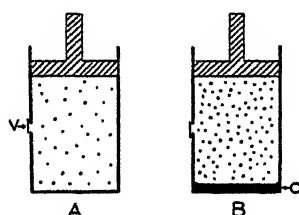


FIG. 3. A mechanical analogue of the changes undergone on activation of the sea urchin egg. For description, see text.

oxygen uptake and the increase in o.i.f. which occurs when the *Arbacia* egg is broken into halves by centrifugal force has been pointed out earlier in this paper. What significance this may have, and whether it is a generally occurring phenomenon, requires further investigation. It has been shown (Shapiro (1941 *b*)) that permeability changes and respiratory changes in the same cell do not necessarily go hand in hand.

V

Calculation of the Point of Intersection

From inspection of Fig. 2 it is evident that the osmotic pressure at which the cell volumes of unfertilized and fertilized eggs from the same batch become equal can be determined analytically. Let the constants for unfertilized and fertilized be indicated respectively by the subscripts 1 and 2; then Boyle's law can be expressed as

$$P(V - b_1) = K_1 \quad (3)$$

$$P(V - b_2) = K_2 \quad (4)$$

THE PERMEABILITY OF HUMAN RED CELLS TO CATIONS AFTER TREATMENT WITH RESORCINOL, *n*-BUTYL ALCOHOL, AND SIMILAR LYSINS

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The loss of K and gain of Na produced by hypolytic concentrations of methanol, ethanol, guaiacol, and resorcinol, as well as by hypolytic concentrations of other lysins, has been described as increasing with time in such a way that what appears to be a new steady state is approached asymptotically (Ponder, 1947 *a, b, c*). In these experiments the curves relating K loss to time seemed to approach steady states which are functions of the concentration of lysin in the system; *i.e.*, they seemed to have the properties of the curve marked *a* in Fig. 1. This apparent flattening-off to an asymptote which depends on lysin concentration leads to the hypothesis that an initially cation-impermeable cell membrane becomes cation-permeable and then cation-impermeable again, or to the hypothesis that the ion-restraining mechanism is something other than a membrane (*e.g.*, a "metabolic pump" or an "ion-binding" mechanism). An extension of the experimental observations to cover much longer periods of time, together with a change in the point of view from which the situation is regarded, leads to a clearer picture of what occurs.

1. Cation Permeability of Red Cells Treated with Resorcinol and with *n*-Butyl Alcohol

A suspension is prepared by washing the cells of freshly drawn heparinized human blood several times with sterile 172 m.eq./liter NaCl, and its volume concentration is adjusted to 0.4. Sixteen ml. of this suspension, cooled to 4°C., is then added to 80 ml. of various concentrations of resorcinol (0.008 M to 0.048 M) dissolved in an isotonic NaCl-NaH₂PO₄-Na₂HPO₄ buffer at pH 6.6,¹ or of *n*-butyl alcohol (0.043 M to 0.172 M) in the same NaCl-buffer, at 4°C. The systems are kept at 4°C. in sterile 100 ml. cylinders; sedimentation is so slow in these vessels that occasional mixing by inversion is sufficient to keep the systems substantially homogeneous. A system containing 16 ml. of the suspension and 80 ml. of the NaCl-buffer is also kept at 4°C., so as to provide a system containing no lysin, with which the systems containing resorcinol or *n*-butyl alcohol can be compared.

After various intervals of time (12 hours, 24 hours, etc.) 5 ml. of the system under observation is removed; the cells are thrown down, and the K content K_p of the super-

¹ The composition of this buffer (pH 6.6) is: 80 ml. of 1 per cent NaCl plus 20 ml. of a buffer mixture consisting of 5 ml. of M/15 NaH₂PO₄ and 15 ml. of M/15 Na₂HPO₄.

natant fluid is measured as a fraction of the initial K content of the cells in 5 ml. of the system (K_0). The values obtained are then plotted against time (Fig. 1).

The three curves in Fig. 1 clearly tend to approach the abscissa as an asymptote, and plotting $\log 1/(1-K)$ against t (inset of Fig. 1) shows that they do so exponentially.² They do not appear to flatten off to a higher asymptote, as does the dotted curve marked a , which is an exponential curve approaching an asymptote at $K = 0.35$. It will be noticed, however, that it would be difficult to detect the difference between the course of the dotted curve a

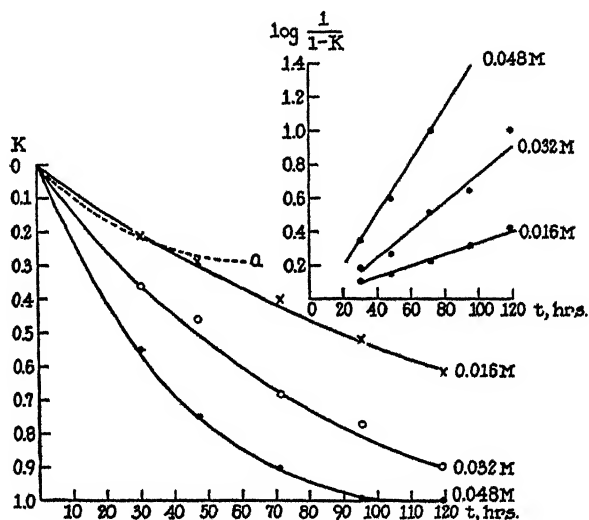


FIG. 1. Loss of K (ordinate) as a function of time, from red cells of a system containing different quantities of resorcinol. Temperature, 4°C. Values of k : 0.0032 for 0.016 M resorcinol, 0.0074 for 0.032 M resorcinol, 0.0145 for 0.048 M resorcinol.

and that of the experimental curve if observations were confined to periods of 40 hours or less (as they were in earlier experiments, Ponder, 1947 *a*).

Although it is not shown in Fig. 1, the curve for the loss of K with time in the system containing isotonic NaCl should be considered also. This curve should be included in the family of curves which show how the K loss varies with time and as a function of the quantity of resorcinol in the system, for it is the member of the family for which the resorcinol concentration is zero. Its course, like that of the other curves of the family, may be described by

$$t = k \cdot \log 1/(1 - K)$$

² To allow for the finite volume of the fluid surrounding the cells, the calculations are carried out with the position of the asymptote set at $K = 0.93$ instead of at $K = 1.0$.

the value of k , however, being very small (0.001 or less). It is not by any means certain, however, that the curve for the loss of K into isotonic NaCl in which no lysin is present is of the same form as that of the curves shown in Fig. 1, and, more specifically, that a new steady state, in which $dK/dt = 0$, is not reached while the value of K is still far from 1.0. This is a point which must be left undecided in the meantime.³

It is only under special conditions that the properties of families of curves such as those shown in Fig. 1 can be demonstrated. If the lysin is too active, the systems show appreciable hemolysis before the K loss is sufficiently great to indicate the position of the asymptote to which the curves are proceeding. On the other hand, if the lysin does not produce a large prolytic loss of K, the curve is so flat that its asymptote is again uncertain, and it is very easy to make the mistake (Ponder, 1947 *a*) of thinking that it follows a course like that of the dotted curve *a* in Fig. 1, and that the rate of K loss becomes zero while the cell K still greatly exceeds that in the surrounding medium. Families of

TABLE I
n-Butyl Alcohol

<i>t</i> <i>hrs.</i>	0.0 (saline)	0.086 M	0.172 M
10	0.07	0.10	0.18
24	0.14	0.20	0.38
48	0.20	0.36	0.70
72	0.26	0.47	0.92

Values given are values of K_p , as a fraction of K_0 , the initial K in the cells.

curves very similar to those shown in Fig. 1 can be obtained, however, for systems containing lysins such as guaiacol or *n*-butyl alcohol, and typical values for K_p for systems containing the latter substance are shown in Table I.

The simplest explanation for our being able to obtain families of curves such as these is that the human red cell is slightly permeable to K and Na even when

³ Many of the curves described by Davson (1937) for the loss of K from the red cells of hypotonic systems seem to flatten-off towards new steady states (like the curve marked *a* in Fig. 1). These observations were restricted to 10 hours or less. Davson accounted for their course by supposing that the red cell swells in the hypotonic medium, that it loses K as its membrane is stretched, and that this loss of K is followed by shrinkage with a cessation of further K losses. There is no direct evidence that the loss of K occurs during swelling only, and it would be interesting to know what the course of the curves for K loss would be if the observations were extended to much longer times. It is by no means unlikely that relatively steady states would be reached, in which case the problem would present some of the aspects alluded to in footnote 4, below.

it is in isotonic NaCl (or plasma), and that the permeability is increased by the presence of hypolytic concentrations of lysin. In all cases the permeability, measured by the constant k , is a function of the concentration of lysin, and it varies in the case of resorcinol from $k = 0.001$ or less when c is zero to $k = 0.014$ when c is 0.048 M. So far as these results alone are concerned, the lysins may be thought of as modifying the ion-restraining mechanisms of the cell in such a way as to create new pathways for the K-Na exchange, the number of pathways being a function of the lysin concentration. The ion-restraining mechanism may be a surface membrane or ultrastructure, a metabolic pump, an ion-binding mechanism associated with the close and orderly arrangement of the substances in the cell interior, or a combination of these;⁴ if it were settled that it consists of a membrane exclusively, the idea of the creation of new pathways through it would be quite a familiar one (the creation of more "pores").

Entrance of K into Red Cells Which Have Previously Lost It.—Systems containing red cells which have lost between 75 and 95 per cent of their initial K into a NaCl-buffer containing resorcinol are obtained in the same way as in the previous experiments; *i.e.*, by allowing the cells to stand exposed to the action of the resorcinol for various lengths of time (50 to 120 hours) at 4°C.⁵ Four 10 ml. volumes of this cell-resorcinol-NaCl-buffer system are transferred to four stout walled tubes. The cells

⁴ The present position may be clarified by pointing out that, *so far as this series of papers is concerned*, the possibility of ion-restricting mechanisms other than a surface membrane arose because the K-Na exchanges were thought to slow down (as in curve *a*, Fig. 1) so that dK/dt became zero at a new steady state value for cell K and Na (Ponder, 1947 *b*; *cf.* Davson, 1937). In *in vitro* systems containing hypolytic concentrations of lysin, it is now doubtful if steady states other than that in which $K = 1.0$ are ever reached; the cation-permeable cells seem to exchange cations until equilibrium is arrived at. Again so far as these systems are concerned, it would be possible, although not necessarily correct, to account for all the phenomena in terms of injury to, and increasing permeability of, an initially slightly cation-permeable membrane. This does not mean that it is no longer necessary to consider the possibility of there being other ion-restraining mechanisms, such as those associated with a metabolic pump or with an orderly internal structure. There is ample evidence that red cells, both *in vivo* and *in vitro*, are not impermeable to cations and yet that substantially steady states are reached and maintained. The normal state of the red cell in plasma, with its unequal distribution of K and Na, is one of these; other examples are to be found in the *in vitro* systems in which K leaves or enters the cell in response to changes in temperature (Harris, 1941), or in association with the development and relief of acidosis (Guest, 1942).

⁵ The results of experiments similar to these have already been described in Ponder (1947 *c*) with the difference that in the latter experiments the K losses from the cells of the systems were much smaller (about 30 per cent of the initial K lost), and that the systems were not kept at 4°C. during the two periods of centrifuging.

are thrown down in each tube, and the K content of the pooled supernatant fluids is determined as a fraction of K_0 , the K initially present in the cells. The cells of the first tube are then packed for K and Na determinations, as already described (Ponder, 1947 b). Ten ml. of isotonic KCl, buffered at pH 6.6, is added to each of the three remaining tubes, and after three suitably spaced intervals of time (usually 30 minutes, 3 hours, and 15 hours) the cells are thrown down, packed, and their K and Na contents determined.

The invariable result of this procedure is shown in Table II, in which the K contents of the cells, in m.eq./liter, are given before and after a 30 minute exposure to isotonic KCl at 4°C. The K gain during this 30 minute exposure varies from 18 to 24 m.eq./liter, and is much larger than the amount of K lost by the cells during any 30 minute period of their exposure to isotonic NaCl containing resorcinol in the initial stages of the experiment, even though the K gradients (inside to outside during the period of K loss, and outside to inside during the 30 minute exposure to isotonic KCl) are comparable. After the first 30 minute exposure to isotonic KCl, the K gain becomes much slower; after 15 hours' exposure the K content of the cells is distinctly less than K_0 , and

TABLE II

Resorcinol M	t	Before KCl wash	After KCl wash	Gain in 30 min.
	hrs.			
0.096	48	21	45	24
0.096	65	12	35	23
0.096	72	8	28	20
0.048	90	16	34	18

Values for K in cells given in m.eq./liter.

the course of the curve relating K gain to time, in so far as it can be judged from the comparatively few points which have been obtained on it, is such as to indicate that it would take a very long time for the concentration of K in the cells to reach the initial concentration K_0 ; the course of the curve, indeed, suggests that K_0 is never reached.

Very similar results are obtained with systems containing *n*-butyl alcohol. Using a system in which the cells have been exposed to 0.086 M *n*-butyl alcohol for 72 hours at 4°C., and in which 47 per cent of the initial cell K is lost (Table I), one finds a gain of 13 m.eq./liter during a 30 minute exposure to isotonic KCl, and a further gain of 8 m.eq./liter when the exposure is increased to 3 hours.

The entry of K during the exposures to isotonic KCl is accompanied by an exit from the cell of some of the Na which had entered the cell to replace the K lost during the early part of the experiment. The quantity of Na which leaves the cell during the exposure to isotonic KCl is approximately the same as the quantity of K which enters, just as the Na which enters during the early part of the experiment, when the cells are losing K into isotonic NaCl, is approximately equal (sometimes a little greater than) the amount of K lost. Throughout these experiments, the K + Na content of the cell seems to remain approximately constant.

Taken in conjunction with the conclusion that all the curves of the families in Fig. 1 and Table I approach the abscissa $K = 1.0$ as an asymptote, these results confirm those previously described (Ponder, 1947 *c*) in showing that the rate of entry of K into red cells which have previously lost K is much greater than the rate of loss of K into isotonic NaCl during the early parts of the experiment; there is no longer any need, however, to distinguish between a portion of the cell K which is mobile and another portion which is not. Now all the K and all the Na can be considered mobile, and their rate of escape from the cell or entry into it can be considered as regulated by the number of exchange pathways and by the value of k ; one need distinguish only between K which has exchanged for Na at the end of time t , and K which has not left the cell in exchange for Na at that time.

There is more difficulty in accounting for the observation that the rate of entry of K into cells which have lost it is greater than the rate of loss of K at any time during the early part of the experiment. There is no indication that the value of the constant k increases with time during the early part of the experiment and while the cells are

TABLE III

<i>t</i>	Resorcinol 0.048 M		Butyl alcohol 0.172 M	
	K_p	V/V_0	K_p	V/V_0
<i>hrs.</i>				
20	0.45	1.08	0.27	0.95
42	0.75	1.10	0.50	0.94
66	0.92	1.11	0.71	0.93
90	0.99	1.14	0.85	0.93

losing K into isotonic NaCl, but its value may increase sharply when the cells are placed in isotonic KCl. The rate of entry of K would then be more rapid than the rate of any preceding loss, and the observation (Ponder, 1947 *c*, Fig. 1) that the K which enters is relatively quickly lost when the cells are again placed in isotonic NaCl is in favor of this explanation. The increase in the value of k would correspond to the creation of additional pathways for a K-Na exchange, presumably as a result of further injury to the cell structure and to further modifications of the ion-restricting mechanisms associated with the structure.

2. Volume Changes

The changes in the volume of the red cells in systems which show the large K losses and Na gains illustrated in Fig. 1 and in Table I can be followed by removing small samples of the systems and determining the volume of the intact cells in Hamburger hematocrit tubes, as already described (Ponder, 1948).

Even almost complete K-Na exchanges are accompanied by only small volume changes provided that only small amounts of hemolysis occur in the systems. This is illustrated in Table III, in which the cell volumes, expressed

as V/V_0 , are shown together with the K losses after various lengths of time at 4°C. for systems containing 0.048 M resorcinol and 0.172 M *n*-butyl alcohol at pH 6.6. In the system containing resorcinol, 99 per cent of the initial cell K was found to be lost after 90 hours, but there was only about 3 per cent hemolysis at the end of this time, and the largest value of V/V_0 was 1.14. In the case of the system containing *n*-butyl alcohol, 85 per cent of the initial cell K was lost after 90 hours; there was less than 1.0 throughout the entire experiment. Cations can accordingly penetrate sufficiently to allow of an almost complete K-Na exchange without any large increases in the cell volume occurring.

The situation is somewhat different, however, when the systems contain quantities of resorcinol large enough to produce considerable hemolysis, as can be seen by comparing the first and the second rows of Table IV. The first row shows the volumes V/V_0 found after various times when the systems contain 0.048 M resorcinol. In this concentration there is virtually no hemol-

TABLE IV

Resorcinol M	30 min.		5 hrs.		20 hrs.		30 hrs.		48 hrs	
	ϕ	V/V_0	ϕ	V/V_0	ϕ	V/V_0	ϕ	V/V_0	ϕ	V/V_0
0.048	0	1.10	0	1.12	0	1.13	0	1.14	0	1.16
0.096	2	1.16	3	1.30	10	1.55	23	1.64	75	1.83

ysis ($\phi = 0$) and, although the K loss is almost complete after 90 hours (*cf.* Table III), the volume changes are quite small ($V/V_0 = 1.10$ to 1.16). The second row shows the volumes V/V_0 after various times in systems containing 0.096 M resorcinol. In this concentration, there is considerable hemolysis ($\phi = 75$ per cent at the end of 48 hours), at least 70 per cent loss of K at the end of 20 hours from the cells which remain intact (*cf.* Ponder, 1948, Fig. 1), and large progressive increases in red cell volume ($V/V_0 = 1.83$ at the end of 48 hours).^{6, 7}

⁶ Systems containing resorcinol are particularly suitable for the investigation of these volume changes because the volume changes in systems containing the higher hypolytic concentrations of resorcinol (0.098 M) are large as compared with those which occur in systems containing hypolytic concentrations of most lysins. In the case of saponin systems, for example, the cells begin to hemolyze when the volume reaches 1.2 V_0 .

⁷ As might be expected, the shape of the cells is fairly well retained after 24 hours in 0.048 M resorcinol at 4°C.; it has already been remarked that these cells undergo disk-sphere transformations and their reversal in much the same way as untreated cells do (Ponder, 1947 *c*). The volume change in these systems is small ($V/V_0 = 1.14$). Cells which have been exposed to 0.096 M resorcinol for 24 hours at 4°C., on

These volume changes are not at all the same as those which would be expected on the basis of the dual mechanism of hemolysis hypothesis or of the colloid-osmotic hemolysis hypothesis. The unlimited swelling and hemolysis observed in higher concentrations of *n*-butyl alcohol (Jacobs, 1947 *a, b*) are certainly not a necessary consequence of cation permeability; it is more likely to be associated with a disintegrating effect of the lysin on red cell structure (the second of the two effects discussed by Ponder (1948)).⁸ It seems to be a mistake to use the assumptions of the dual hypothesis in an attempt to cover the events occurring in lysin-containing systems by the same rules as those which apply to many of the events occurring in simple osmotic systems.

SUMMARY

The form of families of curves relating K loss to time in systems containing hypolytic concentrations of resorcinol and of *n*-butyl alcohol points to the human red cell's being slightly permeable to K and Na even when it is in isotonic NaCl (or plasma), and to the effect of the hypolytic concentrations of lysin being such as to increase this permeability.

The rate of reentry of K into red cells which have lost it is more rapid than the rate of the previous loss. This may be due to the reimmersion of the lysin-treated cells in isotonic KCl producing further modifications of the ion-restricting mechanisms associated with the red cell structure.

The volume changes observed in systems which show the large K-Na exchanges produced by resorcinol and by *n*-butyl alcohol are not the same as those which would be expected on the basis of the dual mechanism of hemolysis hypothesis or of the colloid-osmotic hemolysis hypothesis. Extensive swelling of the red cells occurs only when the concentrations of lysin are large enough to produce considerable hemolysis.

the other hand, are spherical; the volume increase in these systems is large ($V/V_0 = 1.6$ or more). The spherical forms later become prolytic spheres which ultimately hemolyze.

⁸ The difference in viewpoint may be expressed by saying that the dual mechanism of hemolysis hypothesis and the colloid-osmotic hypothesis state that the general "loosening-up" action of a lysin on the cell membrane results in (*a*) an increased permeability to cations, and that this results in (*i.e.*, is the cause of) (*b*) swelling and lysis: the effects (*a*) and (*b*) occur, as it were, in series. The alternative picture presented here is that the general loosening-up action of a lysin produces two effects, (*a*) an increased cation permeability and (*b*) a swelling of the whole cell structure as a result of the lysin's diminishing the cohesive forces between adjacent parts of the cell structure: the effects (*a*) and (*b*) are to be thought of as occurring, as it were, in parallel and as being due to a common cause. The difference between the two points of view is quite important in relation to the model which one would select as best representing the red cell.

APPENDIX

This Appendix contains a summary of the results of a number of experiments which have been carried out to answer a variety of questions which have arisen in the course of these investigations on prolytic ion exchange. The results are negative for the most part.

1. *Electrophoretic Velocity before and after K-Na Exchange.*—The electrophoretic velocity of human red cells before and after treatment with concentrations of ethyl alcohol and of resorcinol sufficient to bring about a 20 to 70 per cent exchange of K for Na was measured in an Abramson vertical cell, and the measurements did not demonstrate any change in velocity. In these systems, some of the cells become crenated and even spherical after a time; the electrophoretic velocity of these was found to be the same as that of the discoidal cells of the system. This result is in keeping with the observation that lysins in general do not affect the electrophoretic velocity (Abramson, Furchgott, and Ponder, 1939).

The cells of systems containing hypolytic concentrations of ethyl alcohol and of resorcinol, when washed and resuspended in their own plasma, usually form rouleaux and sediment at substantially the same rate as untreated cells do. A decrease in the rate of rouleau formation and in sedimentation rate is observed only when the concentration of the lysin is sufficiently great and the duration of its action sufficiently long to produce many crenated or spherical forms; under these circumstances, the decreased rouleau formation can be attributed to the shape change, crenated and spherical cells being incapable of forming rouleaux.

2. *Absence of Hb Denaturation.*—Many lytic substances produce contracture of striated muscle with denaturation of myosin (Mirsky, 1938), an observation which suggests that some of the effects of lytic substances; e.g., the prolytic K losses, may be associated with the denaturation of Hb. Determinations of the O₂ saturation of red cells before and after treatment with amounts of guaiacol and of resorcinol sufficient to produce from 30 to 60 per cent loss of the cell K after 24 hours at 4°C. did not reveal any differences, and so a denaturation of Hb in these lysin concentrations and at this temperature is excluded.

It is not possible, however, to exclude a denaturation of stromatin or of an Hb-stromatin complex, if such exists. It has already been suggested that the normally small cation permeability of red cells can be greatly increased by certain agents which denature proteins; of these *n*-butyl alcohol is one (Jacobs 1947 *a, b*).

3. *Temperature Effects.*—It is conceivable that the human red cell at 4°C. is in a different metastable state from that in which it exists at 25°C. or at 37°C., and that the relative absence of swelling in the presence of large ion exchanges is peculiar to red cells at low temperatures. This interesting possibility remains to be fully explored, but experiments conducted at 25°C. show that large cation exchanges, accompanied by considerable but limited swelling, occur in very much the same way at 25°C. as they do at lower temperatures. In 0.048 M resorcinol, the prolytic K losses tend to be smaller at 25°C. than they are at 4°C. (as they also are in systems containing saponin, Ponder, 1947*a*), and the volume increases also tend to be smaller. There is, however, an increase in the amount of hemolysis observed after long times (48 to 96 hours).

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THE FORMATION OF BACTERIAL VIRUSES IN BACTERIA RENDERED NON-VIABLE BY MUSTARD GAS

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It has been found that *E. coli* B and *Staphylococcus muscae* after treatment with mustard gas [bis(β -chloroethyl)sulfide] can produce phage but apparently can no longer multiply. Most of the experiments were carried out on the *coli* system so that this will be discussed first and in more detail. Table I shows that at a mustard concentration of 0.8×10^{-3} M the phage formation is still 50 per cent or more of the untreated control but the number of organisms which can multiply in a veal-peptone infusion has decreased from 1×10^8 per ml. to about 1×10^3 . When the concentration of mustard gas was $1.25 - 2.0 \times 10^{-3}$ M there were no viable cells and the phage formed from these cells was still 5 to 20 per cent of the untreated control cells. In an occasional experiment after treatment with 1.25×10^{-3} molar mustard the undiluted suspensions showed a few (less than 10) viable cells.

Since the test for viability is the crux of the present problem, an explanation of the tests used is important. Both colony count and culture in liquid media were used in testing for viability. Colony counts were made 24 to 72 hours after plating out on veal-peptone-agar plates; the cells being (a) spread on the surface and (b) mixed with 42°C. veal-peptone-0.7 per cent agar, and then layered onto the plate (2). In the liquid culture serial tenfold dilutions of the organisms were made in rich media and shaken at 37°C. The different media tested were (1) veal infusion-peptone broth; (2) (1) + 0.3 per cent yeast extract; (3) (1) in which normal *E. coli* had been grown from 1×10^8 cells per ml. to 1×10^8 per ml. and then the cells removed by a Berkefeld N filter; (4) difco-brain-heart infusion; and (5) a "complete" media in which were most if not all the vitamins and growth-promoting agents in addition to amino acids, yeast extract, and peptone.¹ Dilutions of untreated cells calculated to contain only

¹ The "complete" medium kindly supplied by Dr. A. C. Braun and R. J. Mandle was made up of the following materials plus water to a liter of solution: 3 gm. of yeast extract, 5 gm. of peptone, 5 gm. of glucose, 0.025 μ g of biotin, 0.01 mg. folic acid, 5 mg. of *p*-aminobenzoic acid, 5 mg. of inositol, and 1 mg. each of thiamin, pantothenic acid, pyridoxine, niacin, riboflavin, and choline. Besides the above, 10 mg. of each of the following amino acids was added to the liter of solution: *dl*-lysine, *dl*-methionine, *dl*-threonine, *d*-arginine, valine, *dl*-histidine, *dl*-phenylalanine, *dl*-leucine, and tryptophane.

2 cells per tube became turbid in 24 hours in all the various liquid media. In a number of test runs using cells treated with varying amounts of mustard, all the various media showed the same number of viable cells for any given treatment. Therefore, only one medium, the veal infusion-peptone broth, was used in the majority of the experiments.

The viable cells present after treatment with $0.8 \times 10^{-3} M$ mustard are not responsible for the phage formation in this suspension for the following reasons:

TABLE I

Tube No.....			I	II	III		IV	
H, final molar concentration....			0 (Saline)	0 ($1.25 \times 10^{-3} M$ hydrolyzed mustard)	$0.8 \times 10^{-3} M$		$1.25 \times 10^{-3} M$	
Cells per ml.....			1×10^8	1×10^8	1×10^8		1×10^8	
Viability tests	Plates	Total cells spread.....	50	50	1×10^4	1×10^4	1×10^7	
		Colonies found.....	10-60	10-60	6	0	0	
	Liquid	Total cells in 5 ml. veal infusion.....	2	2	5×10^5	5×10^4	5×10^7	5×10^8
		Turbidity after 4 days.	+	+	+	-	-	-
Phage formation	Total cell concentration per ml.....		1×10^7	1×10^7	1×10^7	1×10^4	1×10^7	
	Viable cell concentration per ml.....		1×10^7	1×10^7	60	0	0	
	T ₁ phage concentration per ml. in suspension at start.....		2×10^7	2×10^7	2×10^7	2×10^4	2×10^7	
	Phage per ml. (final)...		4×10^{10} *	2.1×10^9	2×10^9	1.8×10^6	4×10^8	
	Phage per cell (final)...		400	210	200	180	40	
	Phage per viable cell...		400	210	3.3×10^7	∞	∞	

* The difference between this value and the corresponding value of Tube No. II is probably accidental since other experiments showed no such difference.

1. There are too few of them. A single viable cell would have to produce about 10,000,000 phage particles to account for the observed result. Normal cells produce about 200 per cell.

2. Lysis and liberation of the phage occurs in an hour or two which does not allow time for much growth of the relatively few *viable* organisms.

3. The yield of phage is approximately independent of the inoculating phage concentration. This would not be expected if the viable cells were multiplying.

4. Diluting out the viable cells does not affect the phage yield per cell.

5. The capacity to form phage does not disappear at higher mustard concentrations where the number of viable cells is zero.

The addition of a few (0.1 and 1.0 per cent) normal cells to a broth suspension of mustard-treated *coli* did not revive any observable number of the latter for

the number of cells that grew up in 3 to 5 hours was the same as in the tubes containing no mustard-treated organisms.

E. coli B received from two different sources, Professor M. Delbrück and Professor A. D. Hershey, have given qualitatively similar results. Most of the

Materials and Procedures for Table I

Preparation of Suspension C.—*E. coli* B were grown from a small innoculum in veal infusion for 10 to 12 hours, then diluted in veal infusion down to $1-3 \times 10^7$ cells per ml. and grown up to 2×10^8 cells per ml. as judged by Klett colorimeter readings. These cells in the log phase of growth were then centrifuged and resuspended in same volume of 0.85 per cent saline - $\mu/100$ pH 8 phosphate buffer.

H.—0.01 ml. mustard gas (M.P. $14.2^\circ\text{C}.$) in 32 ml. saline in a 125 ml. glass-stoppered bottle and shaken hard for 15 seconds. The concentration of mustard was checked by the bromine titration (1).

Tube I.—2 ml. of C + 2 ml. of 0.85 per cent saline.

Tube II.—2 ml. of C left at $27^\circ\text{C}.$ for an hour followed by addition of 2 ml. of H which had stood an hour at $37^\circ\text{C}.$

Tube III.—2 ml. of C + 0.72 ml. saline and 1.28 ml. H.

Tube IV.—2 ml. of C + 2 ml. H.

After all the tubes had stood an hour at $27^\circ\text{C}.$, samples were diluted 1/10 in ice cold veal infusion.

Counts of Cells.—0.5 ml. of indicated cell concentrations in veal infusion was spread on a previously poured veal infusion-agar plate and then incubated at $37^\circ\text{C}.$ Colonies were counted after 24 and 72 hours. Seldom was there any change after 24 hours.

Viability in Liquid Media.—5 ml. of the dilutions in veal infusion containing the indicated cell concentration were shaken in 20 mm. \times 170 mm. tubes at $37^\circ\text{C}.$ for as long as 4 days. Turbid suspensions which exhibited a swirling sheen on shaking were considered positive. Clear tubes indicated no viable organisms.

Phage Formation.—To 5 ml. of the indicated concentration of organisms was added 0.1 ml. of filtered T_1 phage which was 50 times the desired final concentration. The tube was shaken at $37^\circ\text{C}.$ for 1 to 3 hours and then the phage concentration determined by plaque count. A phage control to which no cells were added was analyzed with each experiment.

Plaque counts were obtained after mixing a dilution of the phage with 3×10^7 per ml. log phase *E. coli* B in 0.7 per cent agar-veal infusion and layering 1 ml. of this mixture on a previously poured veal-agar plate (2). Plaques were counted after 12 to 24 hours' incubation at $37^\circ\text{C}.$

experiments have been performed with subcultures of the sample from Professor Delbrück. Besides the T_1 phage used for most of this work, T_{4r} and T_6 phage have also been formed from non-viable mustard-treated *coli*.

The phage-forming capacity of mustard-treated *coli* cells decreases with time. Even in veal broth the phage formation may be virtually zero in an hour or two at $37^\circ\text{C}.$ It is more stable at low temperatures.

In some instances non-viable mustard-treated *coli* can swell or elongate when placed in veal infusion medium. This was observed microscopically and also by turbidity measurements. Table II shows how the capacity for swelling and phage formation varied with increasing mustard gas concentrations. In experiments not shown in Table II the turbidity increase varied several-fold with different concentrations of mustard with no measurable effect on the phage formed per cell. After treatment with 1.5×10^{-3} M mustard there was no change in turbidity and the phage formed was 10 to 20 per cent of the yield with untreated cells. It appears from these various results that the phage formation does not parallel the capacity to swell.

Experimental Procedure for Table II

E. coli B were prepared as described for the experiments in Table I, centrifuged, resuspended in saline - M/100 phosphate buffer pH 8 to a cell concentration of 2×10^8 cells per ml. in one instance and 4×10^8 in a second. These were then mixed with saline and mustard gas, dissolved in saline so the final concentration was that shown in Table II. After an hour at 27°C., each suspension was divided into two equal portions and centrifuged at 7,000 R.P.M. for 10 minutes at 10°C. and resuspended in veal infusion. One portion was shaken at 37°C. and the turbidity determined every 15 minutes in a Klett-Summerson colorimeter employing a 66 filter. The other suspension was inoculated with 2 T₁ phage particles per cell and then shaken at 37°C. till the suspensions had cleared which was usually an hour.

TABLE II
Swelling of and Phage Formation of Mustard Gas-Treated E. coli B

Mustard gas concentration ($\times 10^{-3}$ M).....		0	0.5	1.0	1.5	0	0.8	1.15	Phage control
Swelling	Time of shaking in veal in fusion at 37°C.	Klett turbidity readings (66 filter)							
	0	20	17	17	15	42	36	34	
	1 hr.	65	27	20	16	137	45	34	
	3 hrs.					345	93	38	
	3.5 hrs.	365	84	24	14				
	10 hrs.					530	144	34	
T ₁ phage formation from 1×10^8 cells per ml.									
Plaque count at 1×10^9 dilution.....		80	82	48	11	51	26	12	2
Per cent of zero mustard.....			100	60	14		50	24	4

Respiration studies following treatment with varying concentrations of mustard have been made parallel to estimations of phage formation. Little or no change in oxygen uptake was observed even when the phage formation had

been reduced to 10 per cent or less of normal cells by 2×10^{-3} molar mustard. CO_2 liberation, however, showed a measurable depression at 0.8×10^{-3} molar mustard and was about 50 per cent of normal following treatment with 1.3×10^{-3} molar. The phage formed in these two instances was 70 and 20 per cent respectively of the controls.

It was found that *Staphylococcus muscae* are not as susceptible to the action of mustard gas as the *coli* organism. However, as may be seen in Table III, approximately 98 per cent of the organisms were rendered non-viable for veal infusion media by treatment with 1×10^{-3} molar mustard. This treatment did not depress the phage formation to any appreciable extent. 2×10^{-3} M mustard apparently obliterated the phage-forming mechanism.

Materials and Procedures for Table III

The materials and methods used in the experiments on *Staphylococcus muscae* were the same as for *coli* except that Locke's solution was used in place of saline - M/100 phosphate for the medium in which the mustard treatment was performed. For the general methods of growth of the organism and its phage see reference 3.

TABLE III

Tube No.		I	II	
Final molar concentration of mustard		None	1.0×10^{-3} M	
Cells per ml.		1×10^8	1×10^8	
Viability test	Total cells in 5 ml. broth (4 tubes each)	2	500	50
	Turbidity after shaken 4 days at 37°C.	+	+	-
Phage de-termination	Cells per ml.	1×10^8	1×10^8	10
	Initial phage count per ml.	2×10^8	2×10^8	50
	Final phage count per ml.	2.2×10^9	1.1×10^9	280
	Final phage count per cell.	22	11	28

Over a dozen experiments have been performed on the *coli* system of which only one failed to show phage formation after mustard treatment. In eleven experiments on *Staphylococcus* four failed to show an increase in phage. Since the mustard-treated organisms are quite labile, this may have been responsible for the negative experiments.

DISCUSSION

A number of investigators (3) have presented evidence that phage can be formed by cells which are not actively dividing. Zinsser and Schoenbach (4)

reported a number of years ago that rickettsia multiplied in cells which were not viable but they failed to obtain growth of equine encephalomyelitis under similar circumstances. Anderson (5) concluded that after ultraviolet light treatment *E. coli* would not produce colonies but could form phage; however, no experiments have appeared to substantiate this conclusion.

There can be no doubt from the present results that mustard gas has altered the cells. It is not possible to prove beyond any doubt that the cells are strictly non-viable or dead and cannot be revived for this would require an infinite number of tests, but the media and nutrients used have been varied enough to indicate that they do not multiply when placed under conditions very favorable for phage and cell multiplication. It follows from our results that the capacity for cell division is not necessary for phage formation.

The nature of or the site of the mustard reaction in the cell is not known but one of us has found (6) that bacteria exhibit the same order of sensitivity to mustard as such nucleic acid and nucleoproteins as the pneumococcus-transforming principle and animal, plant, and bacterial viruses. Most enzymes are much less sensitive.

SUMMARY

E. coli B and *Staphylococcus muscae* rendered non-viable by aqueous solutions of mustard gas at pH 7.5 to 8 can still produce phage.

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ACTIVATION OF PASSIVE IRON AS A MODEL FOR THE EXCITATION OF NERVE* ‡

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I. INTRODUCTION

If a piece of passive iron in concentrated nitric acid is touched momentarily with a zinc rod, the iron may become active and the activation may spread from the point of contact over the whole piece of iron. Whether the iron will become active, and if it becomes active, whether it will return to its former passive state depends on the concentration of the acid. There is an intermediate interval of acid concentration in which repassivation will take place. Working with an iron wire at this concentration of acid one may observe a spreading wave front of activation followed by a spreading wave front of repassivation, the whole giving the appearance of a moving spot of activity. Wilhelm Ostwald was the first to note the resemblance of this phenomenon to the transmission of an excitation running along a nerve. In 1900 he suggested to Heathcote, one of his students, that he should investigate this analogy more thoroughly. The papers of Heathcote (1) did not receive the attention they deserve. They contain many interesting facts; e.g., the observation that a wire cannot be reactivated if one touches it with a zinc rod immediately after repassivation. Activation is possible only after the lapse of a certain length of time, in full analogy to the refractory period of a nerve after excitation. The problem was not pursued any further until it was taken up by Lillie (2). Between 1920 and 1935 Lillie published a series of papers in this *Journal* demonstrating a correspondence in the most unexpected details between the excitation of nerve and the activation of passive iron. By analyzing the dependence of the velocity of propagation on the thickness of the surrounding layer of electrolyte his experiments provided definite proof of the rôle of local currents.

It is indeed most astonishing that iron wire and nerve, which from the chemical point of view differ so enormously, function in such a similar way. It does not seem credible that the various functional properties in which the

* The investigations presented in this paper were carried out during the years 1941 to 1946 at the Physico-Chemical Institute of the University of Leipzig. The author would like to express his thanks to Dr. G. Langhammer, Miss V. Hase, and Miss E. Brauer for their active collaboration and valuable help. The present paper also briefly summarizes results obtained in collaboration with others and published during the war years in German periodicals (3, 6, 10, 15).

‡ Translated by M. Delbrück, California Institute of Technology.

two systems resemble each other could be independent and accidental similarities. There is here a most interesting problem from the point of view of reaction kinetics. The existence of a threshold of activation, of a refractory state, of a transmission of activation, of a tendency to give rhythmic reactions, and a suggestion that even the so called accommodation effects are not missing in the model, indicated that all these properties, so uncommon in ordinary chemistry, are in some way related to each other. To get a clearer insight into these relations it seemed necessary to investigate the model more thoroughly from a physicochemical point of view, and such studies are presented in the present paper. The work of previous authors had concerned itself primarily with the *propagation* of activity. The process of *activation* seemed

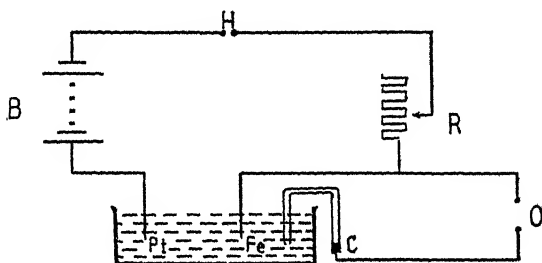


FIG. 1. Arrangement for registering the time course of potential change during a cathodic current pulse on an iron wire in nitric acid. *B* battery, about 60 v.; *H* Helmholtz pendulum (in some experiments with thyatron); *R* resistance, variable from $10^2 \Omega$ to $10^5 \Omega$; *Fe* iron wire, moving with a known speed on a circle of about 2 cm. diameter; *Pt* anode; *C* calomel electrode; *O* direct voltage amplifier and cathode ray oscillograph.

in need of closer study, and for such studies one would prefer to use an electric current of known intensity and duration rather than activation by touching with a zinc rod. It was essential to follow the changes of potential during the reactions as closely as possible and to extend the research to different samples of iron wire, since wires of different composition behave differently. These experiments have led to a better understanding of the remarkable kinetic phenomena mentioned above, perhaps not only for the model, but also for the living cell itself.

II. SHORT ELECTRIC PULSES

Threshold

A schematic diagram of the apparatus used for short electric pulses is shown in Fig. 1. A wire a few centimeters long is dipped into nitric acid. In most of the experiments the concentration of acid was 67 per cent, at which con-

centration the acid has a strong passivating influence on every kind of iron wire we investigated. If intensity and duration of a cathodic electric current pulse are sufficiently great, activation occurs. One then notices a rapid development of gas bubbles at the iron wire, which stops after repassivation. The duration of activity is almost independent of the intensity and duration of the activating pulse and ranges from a tenth of a second to a few seconds. The period of activity is longer, the lower the concentration of acid and, in general, the lower the content of carbon in the iron wire. It may become infinitely long if wires of pure iron in sufficiently dilute acid are used. If intensity and duration of the current are too low for activation, the pulse seems to have no effect at all. Activation, therefore, follows an all-or-none law. For pulses of about 1 msec.

TABLE I

Some Characteristic Data Concerning the Cathodic Behavior of Different Passive Iron Wires in HNO_3 ($d = 1.4$)

(Duration of pulse 2 msec.)

Wire No.	1	2	3	4	5	6	7	8	9	10	11
C, per cent.	0.09	0.25	0.34	0.39	0.41	0.45	0.54	0.61	0.70	0.85	0.92
Threshold, 10^{-6} coulomb/ cm ²	140	100	103	145	153	158	215	228	150	210	200
Rheobase, } ma./cm ²	7	1.0	3.5	2	2.5	4.5	5.6	9.5	10	9.5	12
	(19)	(2.5)	(5.4)	(5.6)	(5.9)	(7.5)	(8.5)	(18)	(9.5)	(18)	(29)
Threshold/rheobase, msec....	20	100	29	73	62	35	38	24	15	22	17
Refractory time, sec.....	<10	95	173	168	138	130	125	85	95	70	70
Accommodation r_{h_2}/r_{h_0} ($\alpha = 500$ msec.).....	20	1.5	1.5	3.5	2.5	3.3	2	6	3.8	7.8	11
Period (60 ma./cm ²), sec....	15	35	46	52	67	—	—	—	—	—	—
Resting potential, vols....	0.99	0.94	0.93	0.95	0.98	0.98	0.98	0.99	0.94	0.98	0.99

duration the effect depends only on the product of intensity and duration (3). We call the quantity of electricity which is necessary to activate 1 sq. cm. of the surface of iron by a short pulse the *threshold of activation* (3).

In Table I a number of such *thresholds* for different samples of wire are summarized.

These values are reproducible with an accuracy of about 10 per cent. For wires 1 to 11 it was found that the threshold is about 20 per cent lower when the wires are moving. We investigated the dependence on acid concentration and found that the threshold in HNO_3 of 56 per cent is about 40 per cent lower than that in acid of 65 per cent. There is an important influence of nitrous acid on the threshold. We have not made quantitative experiments on this influence, but have found that the addition of a small amount of sodium nitrite results in a manifold increase of the threshold. For threshold measure-

ments it is necessary to be certain of the absence of nitrous acid. This is easily accomplished by blowing air through the acid or still simpler by adding urea to the acid, a procedure which exerts no other disturbing effects. The threshold is lowered by the addition of chloride ions, 10^{-3} N Cl^- giving about one-half of the original value and 10^{-5} N Cl^- giving a barely noticeable effect.

Refractory State

Since nitrous acid is formed during activity an increase of threshold is to be expected immediately after repassivation, before the nitrous acid is removed by urea or by aeration. At first sight it seems plausible that the refractory period of the wire could be explained by the presence of nitrous acid developed during activity. As an example we give experiments performed with a steel wire with a C content of 0.91 per cent (Table II).

The expected increase of threshold as well as its gradual decline will be

TABLE II

Dependence of the Threshold on the Recovery Time after Repassivation (Music Steel Wire, 0.91 per cent C) $\text{HNO}_3(d = 1.4)$, Saturated with Urea, 22.5°C .

Time, sec.....	15	20	25	30	50	60	90	100	135	150	240
Threshold, wire not moved, 10^{-6} coulomb/cm ²						1500		1000	800		550
Threshold, wire moving, speed 75 cm./sec., 10^{-6} coulomb/cm ²	1500	1200	1000	925	725		525			350	

noticed. The decline is more rapid if the wire is moving, a fact which also could be expected. However, the great length of recovery time as well as the difference in the behavior of different wires is quite unexpected. If nitrous acid were the sole cause of the refractory behavior the decay of refractoriness should be far more rapid. It should depend on the amount of nitrous acid which had been formed during the period of activity, rising therefore with the duration of the activity. The sixth line of Table I gives the time after which the wire has recovered to a threshold 150 per cent of its ultimate value. There are considerable differences in this refractory time, but no increase of the refractory time with increasing length of the active period is to be noticed. Wire 1, for instance, which has the longest activation period also has the shortest refractory period. One has to look for another, or for an additional cause of the refractory behavior. It may be observed that after repassivation the iron surface is often not as bright as before activation, especially when dealing with wires of high carbon content and long refractory periods. The wires are covered with a visible oxide layer of varying thickness, which disappears gradually during the period of recovery. Once the layer is dissolved, the wire

ceases to be refractory. We believe that the oxide layer constitutes a second cause of refractoriness.

The effect of nitrous acid on refractoriness is easily understood. It can be related to the well known fact that the oxidizing power of nitric acid—or if we put it in another way its reducibility—depends on the presence of nitrous acid. Nitrous acid is the compound which oxidizes and is reduced primarily, nitric acid is reduced only secondarily by the reaction with nitric oxide obtained by reduction of nitrous acid. In the absence of nitrous acid therefore, nitric acid cannot easily be reduced by cathodic treatment and the current then reduces the passivating invisible surface oxide (3, 4). In contrast, in the presence of nitrous acid the current will reduce nitric acid and therefore will be less effective in activating the passive wire.

The thick oxide layer formed during activity may be expected to influence the course of events in two directions. First, the layer may occlude some nitrous acid and protect it against the attack of urea. Second, the layer may use up a part of the electric current for its reduction. Both effects tend to prolong the recovery time. Which of the two effects is the more important has not yet been settled. The decay of the refractory state indicates that there is a superposition of at least two effects. We will return to this question in a later section of this paper.

The refractory period apparently increases if the duration of the testing pulses is increased. This is due to "accommodation" effects which are very pronounced with wires in the refractory state.

Summation and Gildemeister Effect

If we first give a subthreshold pulse and investigate the quantity of electricity necessary to get activation with a second pulse, we find an interesting dependence on the time interval between the two pulses.

Naturally, with a very short interval we find that a lesser quantity of electricity is needed for the second pulse. However, for longer time intervals between the two pulses the sign of the effect becomes reversed, the needed quantity of electricity exceeding that which ordinarily would be necessary for the activation of the wire under investigation. Thus, a subthreshold pulse also engenders some kind of refractoriness. There is here complete analogy to the behavior of nerve, the first effect being the ordinary summation effect, the second effect being one first observed by Gildemeister (4) and later thoroughly investigated by Erlanger and Blair (5). The data in Table III were obtained with wire 2 a. This is a portion of wire 2 (Table I) having a higher threshold than the remainder. As we learned later, it is not very suited for these experiments. Almost any other wire would have given a more pronounced effect, easier to investigate. We believe that this is so because this wire shows very poor accommodation, and, in our opinion, the Gildemeister effect is related to the accommodation effect.

Analogy with Ignition

To explain the existence of a threshold for activation it is useful to compare it with the inflammation point of an explosive mixture of gases. If such a

TABLE III

Summation and Gildemeister Effect. Wire 2 a. HNO_3 ($d = 1.4$), Saturated with Urea. Normal Threshold $150 \cdot 10^{-6}$ Coulomb/ cm^2 First Pulse $60 \cdot 10^{-6}$ Coulomb/ cm^2

Time interval	Second pulse	Difference
<i>sec.</i>	10^{-6} coulomb/ cm^2	10^{-6} coulomb/ cm^2
0.03	130	-20
0.05	140	-10
0.1	145	-5
0.2	150	0
0.5	160	+10
1.0	165	+15
∞	150	0

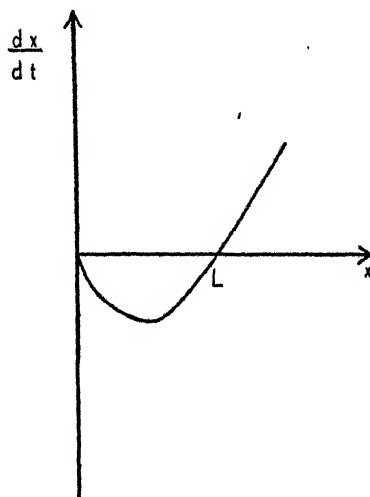


FIG. 2. Diagram showing the analogy between the inflammation point of an explosive mixture (L) and the threshold of activation. x is the difference between the temperature of the mixture and the environment. t = time.

mixture is heated to a temperature just below the inflammation point, and the heat source is then removed, the temperature of the mixture will decrease until room temperature is reached. However, if the mixture is heated to a temperature just above the inflammation point, then the temperature will rise spontaneously, even if the external heat source is removed. In Fig. 2, which illustrates the essential feature of the phenomenon, x means the difference

between the temperature of the mixture and room temperature. The diagram refers to a mixture not exposed to an external source of heat. L is the inflammation point, which is labile.

Assuming similar relations for the threshold of activation we will let the temperature difference x correspond to the degree of activation. There must be a process which is opposed to the activating influence of the electric current pulse. This process corresponds to the cooling of the mixture if left alone. In addition there must be a second process which works in the same direction as the current and which corresponds to the spontaneous heating of the explosive mixture above the inflammation point. The first of the two postulated processes may be attributed to the passivating influence of nitric acid, the second one to the effect of local electric currents which, flowing through the solution from the points already reduced to the points not yet reduced, assist the external current in its activating effect. The existence of these local currents is proved by the fact that activity is transmitted longitudinally, as has been shown elsewhere (15).

Whatever the nature of these reactions, they will take time. If, and only if, the duration of the current pulse is short compared to the time constants of these reactions, should one expect that the effect of the pulse is a function only of the product of intensity and duration of the current.

Oscillograms

Up to this point we have taken the development of gas bubbles as an indication of activity. However, the possibility of observing the changes of potential by an oscillograph gives us another means of detecting activation and this method has the advantage of giving us a clearer insight into what really happens during that process (6). With short electric pulses we get oscillograms like Fig. 3 *a, b, c*, where the course of potential following a short pulse is given schematically. The drop of potential during the electric pulse is so fast, that an almost vertical line is obtained. If the quantity of electricity in the pulse is smaller than threshold, the potential after the pulse rises to the resting potential of $E_H \sim 0.96$ v. For pulses close to the threshold, *i.e.* if a potential of about $E_H = 0.5$ v. (Fig. 3 *a*) is reached, the rise of the potential after the pulse is slower and shows points of inflection. In the immediate vicinity of the threshold, the potential may remain nearly constant for about 50 msec., and then rise rather abruptly. When the threshold is exceeded, the potential, after remaining constant for a while at 0.5 v., drops further to a value of about 0.15 v. indicating that the wire is active (Fig. 3 *b*). The time interval between the application of the pulse and the spontaneous drop of potential depends on the material to be activated and on the intensity of the pulse. Just above threshold this "latency period" has its maximum value. It may be as high as about 100 msec. with wires of very low C content. Fig.

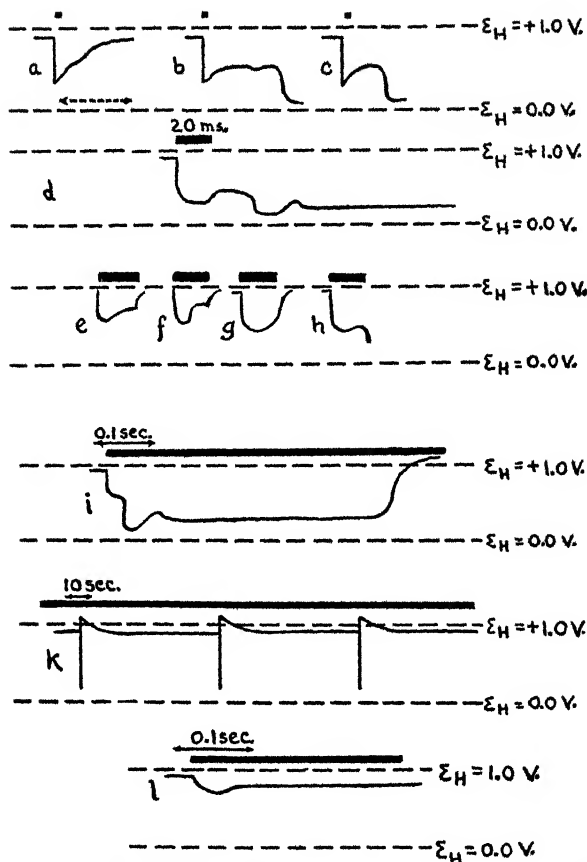


FIG. 3. Time course of the potential E_H of the iron cathode in HNO_3 ($d = 1.4$). The time interval during which the current is flowing is shown by a thick black line above the dotted line $E_H = 1.0$ v.

(a), (b), (c) Duration of pulse 2 msec. (a) Pulse below threshold; (b) pulse barely above threshold; (c) pulse twice threshold.

(d) Activating pulse, duration 20 msec.

(e), (f), (g), (h) Refractory wires, stimulated at various intervals after the conditioning pulse. Duration of second pulse 20 msec. (e) Interval between pulse 15 sec., second pulse does not activate; (f) Interval between pulses 60 sec., second pulse does not activate; (g) Interval between pulses 97 sec., second pulse does not activate; (h) Interval between pulses 105 sec., second pulse does activate.

(i) Activation and repassivation during the flow of a current above rheobase.

(k) Rhythmic activations.

(l) Permanent current below rheobase.

3 c shows the decrease of the latency period if the pulse is doubled. The drop or rise of potential depending on whether or not threshold is reached shows the

existence of the two reactions which we have inferred from the existence of a threshold, one increasing, the other opposing the influence of the electric pulse.

III. ELECTRIC PULSES OF LONGER DURATION

Intensity-Duration Relation

The process which opposes the activating influence of current causes an increase of the quantity of electricity necessary for activation if the duration of the pulse is increased. Fig. 4 shows the logarithm of current density plotted against the logarithm of duration t . A slope of 45° signifies that $i t = \text{constant}$. At about 10 msec. the deviation from 45° becomes noticeable. That means

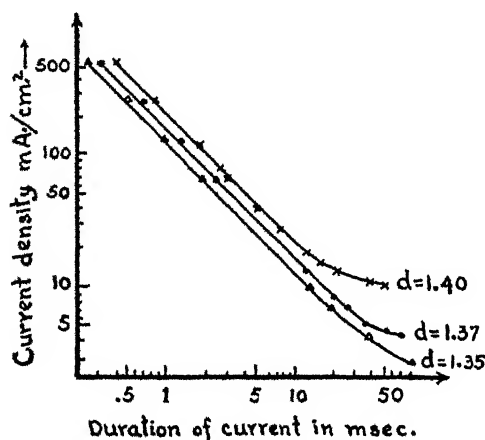


FIG. 4. The current density needed for the activation of an iron wire in its dependence on the duration of the current (three different HNO_3 concentrations).

that the time of 10 msec. is not short compared with the time constant of the passivating counterprocess.

There is a certain current density below which activation becomes impossible. This current density may be called "rheobase" in analogy to the similar behavior of nerve excitation. For HNO_3 ($d = 1.4$) in Fig. 4 rheobase is approximately given by the point with the abscissa $t = 50$ msec. The curves have no asymptotic horizontal for higher values of t , but break off at finite values of t . Rheobases have been measured for different wires and the results are shown in the fourth line of Table I. One of the two figures given for each wire is in parentheses, and refers to measurements obtained with wires which are not yet fully recovered. Accurate measurements of the rheobase of a fully recovered wire are somewhat tedious. The time of rest between two activations has to be at least 20 minutes if the solution is stirred. The figures in parentheses were obtained with waiting times of only 12 minutes during which time the solution was not stirred.

It is of some interest to compare the measurements of rheobase (*i.e.* the lowest activating current density) with those of the threshold (*i.e.* the lowest activating quantity of electricity). A rough parallelism is quite obvious, but there is no exact proportionality. The ratio threshold/rheobase, as shown in the fifth line of Table I, is not constant, it varies between 22 msec. and 185 msec. It is of the order of magnitude of the "chronaxie," the time constant of the counterprocess, but modified by accommodation.

Accommodation

It has been mentioned that the time which corresponds to rheobase in the intensity-duration relation is not infinitely long, but has a finite value analogous to the behavior of nerve. It is the so called maximum utilization time of Gildemeister and is of the order of a tenth of a second. It is possible, therefore, to measure rheobases by means of current pulses of a length of some tenths of a second. The finite value of the maximum utilization time is due to *accommodation*. This effect was revealed by experiments with currents of gradually rising intensity. The dependence of the current density on the time t was given by the equation

$$i = i_0(1 - e^{-t/a})$$

The constant a was varied in the course of different experiments from about 10 msec. to 2 seconds. It was found possible to give i_0 values considerably higher than rheobase without activating the iron. Generally speaking the larger a , *i.e.* the slower the increase of current, the more effective the "creeping-in" of high currents. If we denote the value of i_0 which is just sufficient for activation by rh_a and the rheobase by rh_0 , then the ratio rh_a/rh_0 may become as high as about 20. Undoubtedly even higher values would have been found if our apparatus had been suited for such measurements. With regard to Hill's theory (7) of the accommodation of nerve it is interesting to note that the increase of rh_a/rh_0 with a is not linear, but slows down for higher values of a , and with some wires apparently decreases again. Values of rh_a/rh_0 for $a = 500$ msec. are collected in the eighth line of Table I. The accommodation effects will be discussed in section V.

Oscillograms

It may be inferred from the above that during current pulses of longer duration several secondary reactions take place, some which are connected with the existence of a threshold and others cause accommodation. Both types are revealed by oscillograms. Fig. 3 *d* shows the course of potential during and after an activating pulse of 20 msec. It will be noticed that during the flow of current the potential drop slows down in the vicinity of $E_H = +0.5$ v. and runs almost horizontally at about $E_H = +0.4$ v., showing

that a marked counterprocess is balancing the effect of the current. The secondary effect observed after a short pulse, which is shown in Fig. 3 *a*, is now found superimposed on the primary current effect.

There is one incompatibility not yet fully explained. The counterprocess as revealed by the course of the potential begins within a few milliseconds as shown by the turning of the potential curve. On the other hand for pulses of so short a duration the $i t = \text{constant}$ relation is valid for activation. This indicates that no appreciable counterprocess occurs during the first milliseconds. The only explanation that may be offered is that the potential and the degree of activation may not be connected very closely. Specifically, an increase in the potential may not indicate a repassivation to the same extent as the potential has changed. This assumption does not seem unsound, because the polarization potential could hardly be attributed solely to the reduction of the surface.

Fig. 3 *d* shows the course of potential after the pulse for a period of about 150 msec. The continuation of the oscillograms for such a long time in Fig. 3 *b* and *c* would have led to the same shape of curves. This shape is a rather complicated one, but very well reproducible. After activation, where a potential of about $+0.15$ v. is reached, there is (in HNO_3 , $d = 1.4$) an increase of potential. After passing a maximum the stationary potential of active iron is reached at about $+0.3$ v. A reliable explanation of this behavior cannot yet be given. It is probably connected with the development of large amounts of nitrous acid at the first moment of activation. The shape becomes simpler in more dilute nitric acid.

Since the refractory state is probably in part caused by the presence of nitrous acid it is not without interest to compare oscillograms taken in solutions containing nitrous acid with others taken with wires in the refractory state. Oscillograms in nitrous acid have been published previously (6). In most cases the resting potential is raised somewhat compared with the potential found in solutions free of nitrous acid. The amount by which the potential is raised depends on the nature of the iron. The beginning of the cathodic potential drop does not differ very much from the potential drop in pure nitric acid. Very soon however, the drop slows down and for sufficiently high concentrations of nitrous acid it may even rise during the flow of current, thus showing an apparently "negative" polarization. The higher the concentration the earlier the rise of potential, the minimum passing from right to left on the oscillogram. In the case of very high concentrations the minimum disappears at the left side while the potential remains high, even at high current densities. The explanation of this peculiar behavior of nitrous acid is simple. As mentioned above nitrous acid enables the current to reduce nitric acid thus diminishing the polarizability of the electrode and slowing down the fall of potential. Through the reduction of nitric acid new quantities of nitrous

acid are formed leading to an autocatalytic increase of nitrous acid which is responsible for the reversal of potential change. There remains only the minor difficulty of deciding why at first the presence of nitrous acid does not affect the oscillogram noticeably. This will not be attempted here.

Comparing now the behavior of refractory wires with this influence of nitrous acid, the resting potential will be considered first. In general, the resting potential of these wires is higher than the potential of the normal ones. During the recovery period of the refractor wire this "after-potential" gradually disappears, the wires with a long refractory period giving a long after-potential. The time of decay varies from several minutes to a few seconds depending on the material of the wire and on whether or not the solution is stirred. The amount of the after-potential also depends on the nature of the material, the highest values observed lying around 0.2 v. This fits in very well with the behavior of wires in solutions containing nitrous acid. Fig. 3 *e* to *h* shows oscillograms of refractory wires. They are taken with the same sample of wire at different intervals after activation and show the progressive recovery until activation again occurs. It is interesting to see that there is complete agreement with the shape of the curves taken with nitrous acid, indicating that nitrous acid has something to do with the refractory state. An appreciable amount of nitrous acid must be present on the surface of the wire even 90 seconds after repassivation.

IV. SINGLE AND PERIODIC ACTIVATIONS BY STEADY CURRENTS

To complete the survey we must now discuss the behavior of wires under the influence of steady currents. In this regard the most remarkable fact is that the wires after activation by current generally later again become passive, almost independently of whether or not the current is flowing. The course of potential which is typical for the effect of steady currents is shown in the oscillogram Fig. 3 *i*. Only wires of a very low content of C, which on the whole are more difficult to passivate, sometimes did not return to the former passive state during the flow of current. While repassivation is almost general, the wires show considerable differences in the further behavior during the flow of a current with an intensity greater than rheobase. Every wire investigated by us remained passive if it had become passive during the flow of a current just above rheobase, but at higher current densities and with stirring some wires (*e.g.* wire 1 to 5) again became active followed by a new repassivation. Activation and passivation alternated rhythmically (8). Thus some types of wires (*e.g.* Nos. 6 to 11) may be considered as models of medullated nerve which under the influence of a constant current becomes active only once. The other types of wire are models of non-medullated nerve, *e.g.* of invertebrates, which react rhythmically. In each case, however, a certain limit of current density was found below which no rhythmic activations could be observed. Some-

where P , V , b , and K are respectively the osmotic pressure, cell volume, osmotically inactive fraction of the cell volume, and the Boyle's law constant. The solution gives the x and y coordinates of the intersection of the two functions as

$$\begin{aligned}x &= \frac{b_1 - b_2}{K_2 - K_1} \\y &= \frac{K_2 b_1 - K_1 b_2}{K_2 - K_1}\end{aligned}\tag{5}$$

x being the reciprocal of the osmotic pressure ($1/P$), and y the cell volume, corresponding to the coordinates in Fig. 2. The calculated and observed points of intersection all fell at relative osmotic pressures less than unity (*i.e.*, in hypotonic solutions).

VI

DISCUSSION

What is the significance of these results? The production of insoluble protein on fertilization would lead to a slight decrease in the dissolved molecular content of the cell, and the resulting volume change would be a slight decrease in volume, owing to osmotic adjustment. However, the data show on fertilization an increase in volume for the sea urchin egg, which means, if one considers all volume changes as the consequence of osmotic effects alone, an increase in the number of free particles in solution in the cell. It is probable that this occurs; *e.g.*, the small increase in lactic acid referred to above derives from the breakdown of large carbohydrate molecules; there is also a liberation of echinochrome possibly from some echinochrome-protein complex (Shapiro (1946)), and other enzymatic processes and oxidative changes may lead to an enhancement of the osmotically active content of the cell.

The views incorporated above may be condensed into a simple, admittedly incomplete, analogue. Consider the piston and cylinder shown in Fig. 3. A represents the unfertilized egg, and the constitution of the vapor inside the cylinder is indicated by the dotted array. On fertilization (B), the valve v is opened, and a minute amount of an activator is admitted, which results in a number of physicochemical changes, such that the condensate C appears (analogous in part to precipitated protein) and an increase in the number of gaseous molecules (corresponding to an alteration in the cell's osmotic pressure). The P - V points for the cylinder now fall along a different curve, corresponding to the new Boyle's law constant (*cf.* Fig. 1).

VII

SUMMARY

1. Resting and activated eggs of the sea urchin *Arbacia punctulata* were swollen in hypotonic sea water (60, 70, 80, and 90 per cent), and allowed to attain equilibrium volumes (Figs. 1 and 2).

2. Both fertilized and unfertilized eggs obey the Boyle-van't-Hoff law, but the value for b , the "osmotically inactive fraction" or non-swellable volume, was different for the two, averaging in the cases studied 7.3 per cent for unfertilized and 27.4 per cent for fertilized.

3. On activation, the eggs of the sea urchin undergo a definite increase in total cell volume, of approximately 2.7 per cent.

4. Some evidence is adduced for the possibility that the alteration in cell volume and in o.i.f. may depend upon the species in question.

5. A parallelism between change in b and alteration of respiratory metabolism in *Arbacia*, *Chaetopterus*, and *Arbacia* fragments is pointed out. This requires further investigation in other species to establish generality.

6. Equations for the calculation of the point at which osmotic pressures and cell volumes are identical for unfertilized and fertilized eggs are included.

7. A mechanical analogue of the phenomena is introduced (Fig. 3).

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times, in the transition region between rhythmically repeated and single activation we succeeded in producing an intermediate reaction consisting of a finite number of repeated activations analogous to the observations which Arvanitaki (9) made in her examinations of non-medullated nerve. The range of current density between the first single activation and the region of rhythmic activations is very different with different wires. For wire 2 it is about 0.25 ma./cm.² (rheobase at about 1 ma./cm.²), while for wire 5 it runs up to 40 ma./cm.² (rheobase at about 2.5 ma./cm.²). There is also an upper limit for the rhythmic behavior, at about 300 ma./cm.² for wire 2, and at about 60 ma./cm.² for wire 5. Thus the interval for rhythmic reactions is much smaller for wire 5 than for No. 2. Wires of a higher content of carbon than that of No. 5 showed periodical behavior only at temperatures above room temperature and with concentrated nitric acid ($d = 1.4$). The length of the time during which the wire is passive and recovering is given for different wires in Table I. It effectively determines the length of the period, since the duration of the active state is only a fraction of a second. The oscillogram Fig. 3 *k* may serve as an example of a rhythmic reaction, each peak corresponding to the oscillogram Fig. 3 *i*.

V. CORRELATION BETWEEN THRESHOLD, ACCOMMODATION, REFRACTORINESS, AND RHYTHMS (10)

The Kinetic Equations

We have now gathered the material which will enable us to discuss the interrelations between threshold, accommodation, refractoriness, and rhythmic behavior. Beginning with the last mentioned phenomenon we recall how undamped electric oscillations are generated. There must be an influence which compensates for the natural damping of the oscillations. This self-exciting effect is caused by a so called "negative resistance" in the system, which means that in a part of the system an increase in voltage goes parallel with a decrease of current intensity I . In a system containing in series a condenser C , a self inductance L , and a resistance R with a voltage $E_R(I)$, we have the differential equations

$$\begin{aligned} dE/dt &= -I/C \\ dI/dt &= E/L - E_R(I)/L \end{aligned}$$

where E is the voltage across the condenser. This system has the required properties if at least one region shows a rise of $E_R(I)$ for decreasing I . Simplifying our chemical problem we may assume that its kinetics, too, is determined a set of two similar simultaneous differential equations of two variables x and by y , which define the state of the system. We will write them in the form

$$\begin{aligned} dx/dt &= f(x, y) \\ dy/dt &= g(x, y) \end{aligned} \tag{1}$$

In order to obtain solutions of the oscillatory type from a differential equation of the first order there are required at least two variables defining the state of the reacting system at each moment, as, *e.g.* position and momentum in mechanical oscillations, or voltage and current in electrical oscillations. In our system choose the "degree of activation" as one of these variables, for which we may take the electrochemical potential as a rough measure. If we look at Fig. 2 which applies to conditions where no current is flowing we find that above the threshold dx/dt is positive and rises with rising x . This is due to the spontaneous increase of active areas by local currents, and is responsible for the existence of a threshold. We can put this auto-catalytic increase of x into analogy to the increase of dI/dt with rising I in the equation of electrical oscillation. Thus an autocatalytic reaction corresponds to the "negative resistance" and the lability caused by it is the reason for the existence of a threshold and it is also a necessary, though not a sufficient condition for the occurrence of rhythmic reactions.

We must now seek to determine the second independent variable, y , by which the state of the wire is to be described. We will choose the refractoriness of the wire as measured roughly by its threshold (in the inactive state). Since refractoriness is generated in a wire which is becoming active, dy/dt is positive when x is positive; on the other hand the rate of increase of activity, dx/dt , produced by the current, is diminished by the refractoriness y . In a qualitative way the interrelations between the two differential equations are thus given. From this point of view we have to extend the scheme of Fig. 2. First we must realize that x cannot rise indefinitely. Therefore the curve must turn and after passing a maximum must reach the abscissa as shown in curve *c*, Fig. 5. The physical meaning of this decrease is that local currents lose their activating power when the whole surface is active. The S-shaped character of the curve is due to the counteraction of the passivating nitric acid, a counteraction which at low activities outbalances the activating influence of local currents. In Fig. 5 the curve *a* does not begin at the origin of the coordinate system, but at a point very close to it. This is to account for the fact that even passive wires, represented by the point P_0 , show a little dissolution of metal and therefore a small amount of activity.

If an external current is applied the curve is raised and is shaped like curve *b*. Neglecting the influence of x on y one should expect that rheobase is defined by a curve, the minimum of which is touching the abscissa.

Below this current density the curve will cross the abscissa in a stable point which is approached asymptotically from $x = 0$, representing a low degree of activation; after cessation of the current, the system will return to the point P_0 . On the other hand full activation would be obtained above this critical current density and if the current is interrupted after the system has passed the abscissa of L , given by the dotted line, spontaneous activation will occur

along curve a . If the current density and the corresponding lift of the curve are so high that the values of dx/dt for x between O and L may be considered as constant, we have the region where $it = \text{constant}$ may be applied to the intensity-duration relation.

However, to account for accommodation, refractoriness, and rhythms we have to consider the influence of x on y and how y modifies the position of the

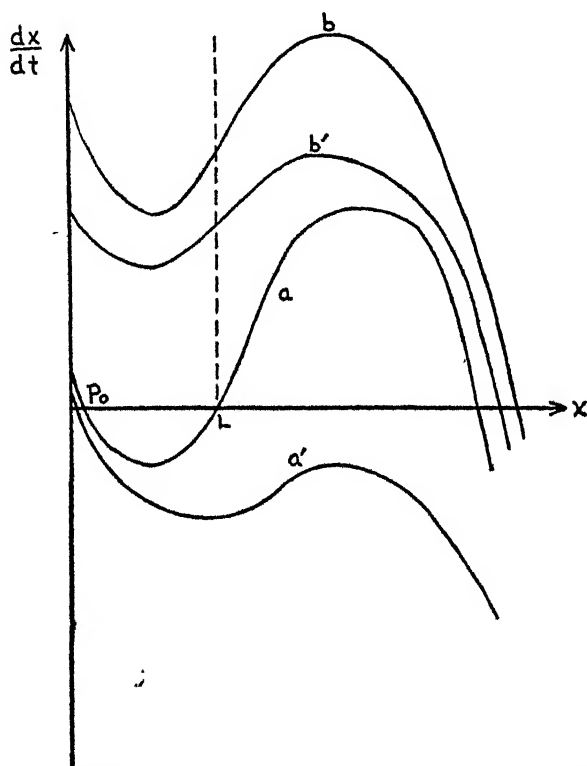


FIG. 5. The rate of change in activity in its dependence on the activity. Curves a and a' , no current, a , no refractoriness, a' , refractory. Curves b and b' the corresponding curves for current densities above rheobase.

point for which $dx/dt = 0$. We assume that nitrous acid and oxide layer are formed not only on the fully active surface of the iron—a factor which causes refractoriness after repassivation—but also on parts of the surface which are only partially reduced, thus causing accommodation.

The curves a and b in Fig. 5 refer to the non-refractory state with $y = 0$. Higher values of y will lower the curves, the new positions being shown by the curves a' and b' . The change of shape of the curves is due to the fact that the activating effect of local currents is also reduced. Thus during the process of

activation by a current of constant intensity the point which is indicating the state of the system is shifting continuously from a higher curve to a lower one corresponding to the increase of the refractoriness y .

The x y Diagram

For a survey of the whole variety of changes of state under the influence of direct currents, it is advisable to use a diagram with the coordinates x and y , and to insert at each point the direction dy/dx of the change, obtained by dividing the two differential equations (1). Qualitatively $f(x, y)$ can be read from Fig. 5. For the construction of the curves dy/dx , however, $g(x, y)$ must first be obtained. From the decay of refractoriness after repassivation we know that dy/dt is negative for very small values of x and tends to zero if y itself is very small. We know, furthermore, that the decay of refractoriness is very slow compared to the development of refractoriness during the short period of activity. Thus the absolute values of dy/dt are generally very much higher if positive than if negative. The change from small negative values of dy/dt to large positive values takes place within a small interval of x values in the vicinity of the threshold. Finally we know that in case of high values of refractoriness the tendency to removal of nitrous acid and of oxide layers will always be stronger than in case of lower values. These data furnish a sufficient basis for a qualitative picture of $g(x, y)$ and therefore also for the construction of a diagram in x, y coordinates for different current densities.

Let x_0 signify the maximum value which is attainable for x (full activity, *i.e.* completely reduced iron surface) and y_0 the maximum value of y ; *i.e.*, the highest grade of refractoriness. Neither dx/dt can be positive if $x = x_0$, nor dy/dt if $y = y_0$, and we obtain the following diagrams (Figs. 6 to 9). In these diagrams the curves $dx/dt = 0$ (Fig. 6, curves HF_1 and LF_2 , Figs. 7 to 9, curves HF) and $dy/dt = 0$ (curves EG) have been inserted. According to our assumptions the curve $dx/dt = 0$ hits the border lines of the square between D and C and between A and B , whereas $dy/dt = 0$ hits between A and D and between B and C . The curve $dx/dt = 0$ may hit the same border line several times because of its S-type shape (Fig. 6). We will first discuss the case when there is only one point of intersection on each side of the diagram (Figs. 7 to 9). We denote these points respectively by H, F, E, G . There must be at least one point of intersection of the curve $dx/dt = 0$ with the curve $dy/dt = 0$. We will assume throughout that there is only one such point and denote it by P (P_0 in Fig. 6). The two curves divide the diagram into four sectors. In going from one sector to an adjacent one dx/dt or dy/dt will change signs. The signs of these derivatives are such that in the lower left sector $A F P E$ the point which represents the state of the system moves upwards from left to right. Correspondingly, in sector $B F P G$ the point moves upwards from right to left, in sector $C G P H$ downwards from right to left, and in sectors

$D H P E$ downwards from left to right. The curve $dx/dt = 0$ is crossed by the path of the point in a vertical direction, the curve $dy/dt = 0$ in a horizontal direction, as indicated by short arrows. Since the changes of y are generally slow compared with the changes of x in the same time, the paths for the most part run almost horizontally. This applies particularly to the left side of the curve $dy/dt = 0$, where dy/dt is negative. Only in the neighborhood of the curve $dx/dt = 0$ does dy/dt become comparable in magnitude to dx/dt , and the path show a considerable deviation from the horizontal direction.

Threshold.—We will now discuss the conditions for different current densities. When there is no current we get a diagram like Fig. 6. It differs from the

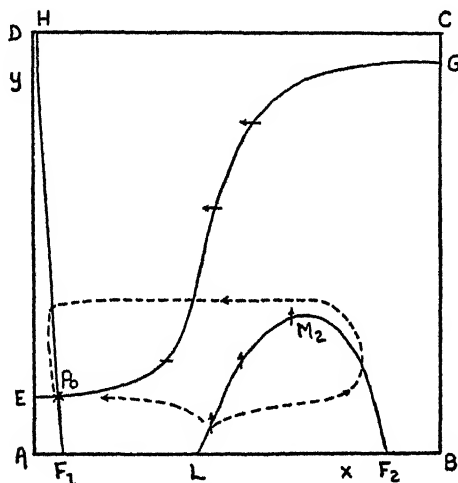


FIG. 6. xy diagram. x activity, y refractoriness. No current. Along HF_1 and LM_2F_2 $dx/dt = 0$, along EG $dy/dt = 0$. The arrows and the dotted lines show how the system changes when no current is flowing.

scheme just discussed by the fact that the curve $dx/dt = 0$ hits the border line AB three times, in F_1 , L , and F_2 . Thereby a new area is formed below the curve LM_2F_2 , in which the direction of path behaves like the direction in the area AF_1P_0E ; i.e., in that region activation is increasing even when no current is flowing. It is the region of spontaneous activation. If after interruption of a current the state of the wire is given by a point lying in the region LM_2F_2 activation takes place. The curve LM_2 indicates the condition of threshold, P_0 the stable resting point of low activity and low refractoriness. For sufficiently low current densities we obtain conditions which are qualitatively illustrated by Fig. 6. We will assume that the only influence of the current is the lifting of the curve $dx/dt = 0$, the curve $dy/dt = 0$ remaining unaffected; this is certainly a crude simplification the value of which may be

doubted. The environment of P_0 is given in Fig. 7 on a bigger scale. The density of the current is below rheobase, and starting from P_0 no activation is possible because the sector $L M_2 F_2$ cannot be reached. The path is marked by a dotted line. We obtain an increase of activity and later a small decrease asymptotically approaching the point P . This result is fully verified by oscillograms with currents below rheobase where the potential is found to go through a minimum (Fig. 3 I). The retrogression of the potential is caused by the formation of nitrous acid.

Accommodation.—The rise of the dotted line in Fig. 7 near P shows the increase of refractoriness; *i.e.*, accommodation of the wire for current densities below rheobase. It also shows that the observed rheobase must be larger than

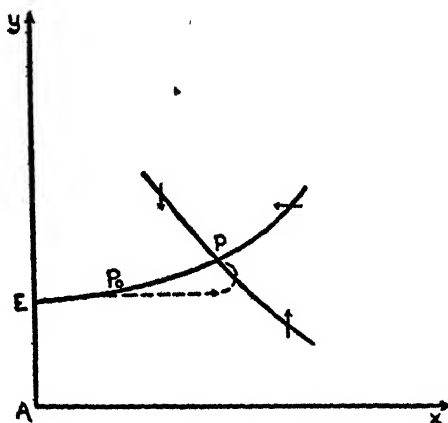


FIG. 7. Part of an xy diagram for current densities below rheobase. P_0 is the resting point of the system when no current is flowing. P the intersection point of the curves $dx/dt = 0$ and $dy/dt = 0$.

the theoretical value without accommodation; *i.e.*, without the rising of the dotted line. The parallelism between rheobase and the ability for accommodation, which our experiments had indicated, suggests that the value of the rheobase is determined at least partially by accommodation. It may be read from the figure that the duration of a rheobasic current needed for activation is finite, because for this current the time to reach the sector $L M_2 F_2$ is finite. Since these diagrams apply to constant currents only they are not appropriate for treating accommodation effects observed with slowly increasing currents.

It turns out that the underlying idea is not so very different from Hill's assumptions in his formal theory of accommodation (7). In both cases the state is described by two variables and the change of state by two simultaneous differential equations. In Hill's theory, as in the one here described, the rise of threshold during activity is assumed to correspond to the increase of refrac-

toriness. There is a difference in so far as Hill's theory attributes the increase of the threshold during accommodation to a higher value of activity which has to be reached while we attribute it to a slower rate of increase of activity. Perhaps this is a question of minor importance. The quantitative results obtained in Hill's theory are essentially due to two simplifications of the problem. First, Hill's theory does not attempt to treat the relation between accommodation and the phenomena occurring during and after activation. This however, is precisely the problem dealt with in these pages. Second, the differential equations of Hill's theory are set up on the assumption that the time constant which regulates the rise of threshold during activation is the same as the time constant

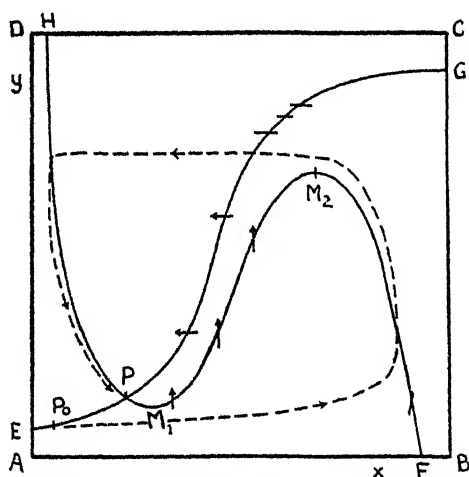


FIG. 8. xy diagram for current densities barely above rheobase. Single activation. Fig. 8 is obtained from Fig. 6 by raising the curves HF_1 and LM_2F_2 . The notations and the meaning of the lines are the same as in Fig. 6.

of its spontaneous decrease. This should not be expected *a priori* and it does not apply to the types of wire studied in our investigations. There is no proportionality between the time constant α of exponentially rising currents which can creep in and their final intensity i_∞ . The existence of such a proportionality would follow from Hill's deductions, as he himself has shown.

Lower and Upper Limit of Periodicity.—If the current density is raised above rheobase we obtain a diagram as shown in Fig. 8. The dotted line again describes the changes of the state of a wire. The rate of increase of activity x slows down when the dotted line comes into the neighborhood of the minimum value M_1 , but rises again when leaving it. At higher values of x the refractoriness begins to increase and x attains its maximum value crossing the curve $dx/dt = 0$ vertically. This is exactly the course of the potential as observed

in the oscillograms (Fig. 3 *i*). Now the refractoriness increases and finally near the maximum M_2 becomes so high that the passivating influence of the nitric acid surpasses the activating influence of the current and repassivation occurs. Curve $dx/dt = 0$ is reached again and must be crossed downwards in a vertical direction. Now the wire shows slow changes in the region of small values of dx/dt and dy/dt and approaches the stable point P asymptotically. That is the case when only a single activation is found under the influence of a constant direct current. On further increasing the density of the current, the picture may remain unchanged qualitatively, as long as the curve EG crosses the curve $dx/dt = 0$ between H and M_1 . However, for higher current densities it may

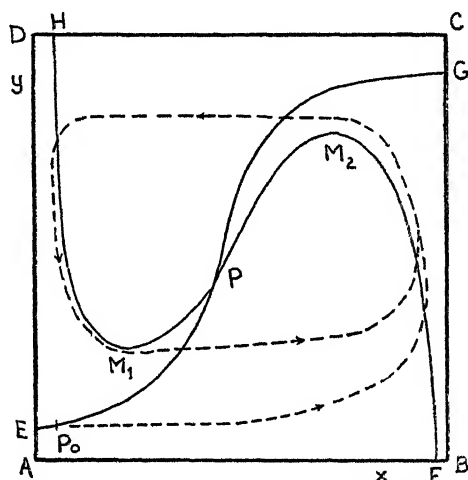


FIG. 9. xy diagram for higher current densities. Rhythmic activation. The notation and the meaning of the lines are the same as in Fig. 6.

happen that the intersection of the two curves lies between M_1 and M_2 (Fig. 9). Then rhythmic activations are obtained in the following manner. The intersection point is a labile node. There is a closed curve to the outside and inside of which the paths of the system cling like spirals. In Fig. 9 the outside and the inside spirals would run counterclockwise. Since dx/dt may be considered large compared with dy/dt (except near the curve $dx/dt = 0$), this closed curve is formed by the two horizontal tangents in M_1 and M_2 and by two curves which run close to the left side of $H M_1$ and close to the right side of $M_2 F$. A path beginning at P_0 is inserted in Fig. 9 (dotted line). We might add that for a finite number of repeated activations, the intersection point P would be expected to fall in the immediate neighborhood of M_1 , but on the left side of it between H and M_1 .

On increasing the current density and further raising the curve $dx/dt = 0$, the intersection point P may wander beyond M_2 . At this moment the periodic

activations would cease, thus explaining the upper limit of current density giving rhythmic activations, which, in fact, was always observed. It may also happen that two new intersection points appear, one of which may lie between M_2 and F . This would have a similar effect with regard to rhythmic activations.

The method developed here for the discussion of cathodic activation of passive iron is similar to that used by Friedländer (11) and by Kirschstein (12) for the discussion of relaxation oscillations. The idea that rhythmic activations in biological systems should be considered as relaxation oscillations is not a new one. Bethe (13) stressed this idea long ago and a more precise development has been undertaken by van der Pol and van der Mark (14). The aim of our discussion is merely to determine how the differential equations of chemical kinetics can lead to relaxation oscillations. The result is that the assumptions which we must make in order to understand periodic reactions also lead to an understanding of the existence of a threshold, of accommodation, and of refractoriness. We have not here discussed one very characteristic phenomenon of our system, the propagation of activity along a surface. In an earlier communication (15) it has been shown that the lability which gives rise to the existence of a threshold and to rhythmic reactions also provides the necessary condition for the propagation of activity without decrement along the surface. There is the same relation between the inflammation point and flame propagation as between the threshold of activation and the transmission of activity. It could be shown that the velocity of stationary propagation of activity is in good agreement with the value calculated from the observed magnitude of the threshold. However, the more general non-stationary case, the origination of the excitation wave, for example, or its disappearance, or the propagation of activity with decrement, are not equally well understood. Experiments with iron wires in chromic acid have shown that in certain cases the intensity of response (decrease of potential) is not independent of the stimulus (current pulse), in other words, the all-or-none law is not valid here. On the other hand, in these cases the spreading activity generally showed a decrement. A correlation between the two facts, therefore, has to be assumed.

The existence of *spontaneous* rhythmic activations seems to be connected with the possibility of propagation. For this reason spontaneous activations have also not been discussed here. We do not wish to imply that all spontaneous activities are due to waves emitted from an active center and that an inhomogeneous surface is essential for their origination, but certainly that is usually the case and also ought to be expected theoretically.

VI

SUMMARY

The activation by cathodic polarization of passive iron in concentrated nitric acid ($d = 1.4$) has been investigated.

1. For short current pulses (1 msec. or less) a transient activation occurs when the product of current density and time exceeds a certain value. This limiting value is here designated as the "threshold." It is of the order of magnitude of 200×10^{-6} coulomb/cm.².

2. After activation and repassivation the threshold is temporarily several times higher than before. This "refractory state" is due to the presence of nitrous acid and of oxide layers. The return of the threshold to normal values occurs in seconds or minutes, depending on the variety of iron wire.

3. Immediately after a subthreshold current pulse the threshold is reduced (summation). However, if the second pulse occurs a certain interval of time after the first the wire exhibits a certain degree of refractoriness (Gildemeister effect).

4. Oscillographic measurements reveal the existence of a latent period between the application of the stimulating pulse and the establishment of the active state. The duration of this latent period depends on the strength of the current pulse.

5. There exists a minimum current density (rheobase) below which no activation occurs however long the current is applied. Depending on the variety of iron used this current density varies between about 1 and 10 ma./cm.². To produce activation a current of rheobasic strength does not have to be applied for an infinite time but only for about 100 msec. (maximum utilization time). Activation becomes manifest some time after termination of the activating pulse.

6. With currents of slowly increasing strength it is possible to reach current strengths several times higher than rheobase without obtaining activation (accommodation). Accommodation to a large extent depends on the variety of iron used. Details are given for currents increasing with a time constant of 0.5 second.

7. Potential measurements on wires in the refractory state show the existence of after potentials. Wires in the refractory state which are cathodically polarized show peculiar oscillograms. Both types of experiments point to the formation of nitrous acid as an essential element in the course of events.

8. With current densities only slightly above rheobase all wires exhibit simple activations only. With higher current densities certain types of wires exhibit periodic activations. The range of current densities in which such periodic activations occur varies with the type of wire. The lower limit is sometimes quite close to the rheobase.

9. A theory of periodic activations is presented which is modelled on the theory of self-excitatory electrical oscillations. As variables describing the state of the wire, the "degree of activation" and the "degree of refractoriness" are introduced. In the physicochemical system an autocatalytic process corresponds to the "falling characteristic" of electrical oscillations. The theory

leads to a rational view of the interrelations between threshold, rheobase, accommodation, refractoriness, and rhythm. The phenomena of conduction are not discussed here but their relation to the theory is briefly touched upon.

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NITRATE REDUCTION AND ASSIMILATION IN CHLORELLA*

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Nitrate is a common starting point for nitrogen assimilation in many micro-organisms and in higher plants. The extensive literature on the reduction and assimilation of nitrate has recently been reviewed by Burström (1945). Unfortunately no comprehensive generalization of the mechanism of nitrate reduction is yet possible. The present study of nitrate reduction and assimilation in *Chlorella* has bearing both on the general features of the process and the rôle of nitrogen in algal metabolism.

Nitrate reduction in *Chlorella* has been studied by Warburg and Negelein (1920) under conditions specifically chosen to simplify certain features of the process. The cells were immersed in a "nitrate mixture" containing 0.1 M KNO₃ and 0.01 M HNO₃ and studied both at high light intensity and in darkness. In the dark the rate of oxygen uptake increased about 40 per cent when the cells were transferred from Knop's solution to the nitrate mixture; but at the same time the rate of carbon dioxide output increased still more to give a CO₂/O₂ quotient of about 1.5 and measurable amounts of ammonia were excreted. The carbon dioxide production in excess of the oxygen uptake was labelled "extra-CO₂." After a period of several hours the molar ratio of ammonia to extra-CO₂ approached a value of 0.5, compatible with the equation



Initially, however, and particularly in nitrogen-deficient cells, the NH₃/extra-CO₂ ratio was much lower than 0.5 and it was inferred that the excretion of ammonia was suppressed by attendant assimilation to cellular materials. Ammonia was the only nitrogenous excretory product although under anaerobic conditions nitrite was produced and rapidly proved toxic.

Warburg and Negelein also exposed *Chlorella* to photosynthesis-saturating illumination. Cells in the nitrate mixture then showed an excretion of oxygen and ammonia even though no carbon dioxide was provided. In light the extra-O₂ and ammonia were excreted two to three times as rapidly as the extra-CO₂ and ammonia produced in the dark. Relations of nitrate reduction to respiration and photosynthesis were studied by comparing the effects of cyanide and urethane on the three processes. The acceleration of nitrate reduction by light was explained in terms of an increased permeability to HNO₃ allowing acceleration of equation (1), the extra-CO₂ being converted photosynthetically to extra-O₂. Alternative explanations have been suggested by Rabinowitch (1945).

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The present paper extends the study of nitrate reduction to those conditions which Warburg and Negelein chose to avoid; *i.e.*, conditions of normal growth in which the product of nitrate reduction is consumed in subsequent nitrogen assimilation. The organic materials of *Chlorella* contain approximately 10 per cent nitrogen and 50 per cent carbon. In an organism with such a high nitrogen requirement the reduction of nitrate should have observable effects on the CO_2/O_2 gas exchange ratio even during normal metabolism. Upon this expectation the present work is based.

EXPERIMENTAL

Chlorella pyrenoidosa (Emerson's strain) was grown in a continuous culture apparatus which provides uniform experimental material day after day (Myers and Clark, 1944). Knop's solution containing KNO_3 , MgSO_4 , and KH_2PO_4 with added iron and microelements was used as the culture medium as previously described (Myers, 1946). The cultures were aerated with 4 per cent carbon dioxide, maintained at a temperature of 25°C ., and illuminated at an intensity of 90 foot-candles by tungsten lumiline lamps. The population density was maintained at about 2.5 c.mm. cells/ml. At such a high population density there is considerable mutual shading of the cells. The observed growth rate is less than the maximum possible rate and corresponds to that observed at an intensity of 40 f.-c. in thin suspensions (*cf.* Myers, 1946).

Metabolic gas exchange was measured by the Warburg technique at 25°C . in Knop's solution at pH 4.5. Variation of the nitrogen source was accomplished by replacing the 0.0124 N potassium nitrate with equinormal concentrations of ammonium sulfate or potassium sulfate or by adding equinormal ammonium sulfate. For the light experiments an intensity of about 40 f.-c. was selected in order to approximate growth conditions. Illumination was provided by tungsten lamps and copper screen filters, by tungsten lamps operated at reduced voltage, or by a grid of neon discharge tubes. Voltage supply to the light source was held constant to ± 1 per cent by a voltage stabilizer.

The measurement of the CO_2/O_2 quotient at low light intensities involves considerable difficulty. One problem lies in obtaining perfectly uniform illumination of the duplicate vessels required for the indirect method. No practical means could be found to obtain this uniformity directly. The following procedure was therefore devised: at a time precisely in the middle of each experiment the positions of the two flasks and manometers of a given pair were exchanged. While this procedure tends to average out any differences in intensities between the two positions, its final effectiveness depends upon the constancy of the light source. The output of the neon grid was remarkably constant. With tungsten illumination the light intensity in any one position did show a short period variation of about ± 1 per cent which is characteristic of the voltage stabilizer; however, these variations are averaged out by allowing a 30 minute period before and after change in the flask position.

A second problem at low light intensities arises from the requirement of rather dense suspensions in order to obtain a measurable rate of gas exchange. About 12 c.mm. cells/flask were commonly used. This results in absorption of a large fraction (about 60 per cent) of the incident light and adds the requirement that the two

flasks of a given pair expose equal cross-sectional areas to the illumination. An over-all check on the precision of the method is demonstrated in the fact that the flasks could be used in various combinations and positions without effect on the observed CO_2/O_2 ratios.¹

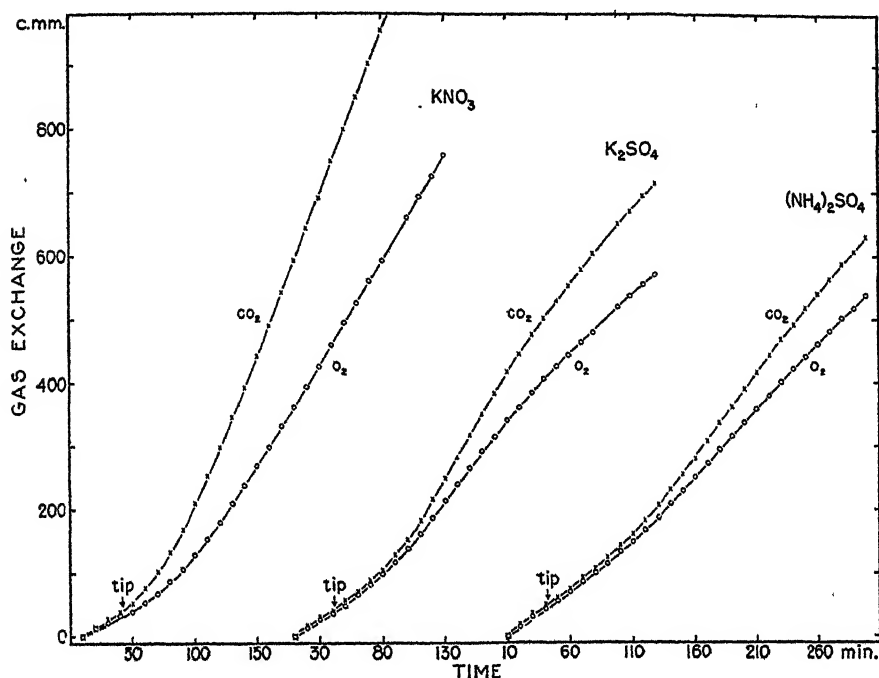


FIG. 1. Oxygen uptake and carbon dioxide production during glucose respiration of growing cells in the dark. The KNO_3 of the Knop's solution in which the cells were suspended was replaced in the second and third cases by equinormal amounts of K_2SO_4 and $(\text{NH}_4)_2\text{SO}_4$, respectively. Zero time for each set of curves represents the beginning of the manometric experiment. Glucose was added from sidearms by tipping at the times indicated.

Nitrate Reduction during Glucose Assimilation in Darkness

Chlorella grows rapidly in the dark with glucose as the only source of carbon. Fig. 1 illustrates the course of gas exchange when glucose is added to cells suspended in Knop's solution containing potassium nitrate or ammonium sulfate as the nitrogen source or without a nitrogen source (KNO_3 replaced by K_2SO_4). The cells had previously been growing photosynthetically in Knop's

¹Two of the CO_2/O_2 quotients cited in a preliminary report of this work (Myers and Cramer, 1947), now known to be in error, were obtained before the above precautions were put into operation.

solution with potassium nitrate as the nitrogen source. Examination of Fig. 1 shows that the highest and most constant rate of gas exchange is attained on nitrate, to which the metabolism of the cells had already been adapted. With ammonia as a nitrogen source the gas exchange is less rapid and shows a gradual decrease with time which may be related to a marked decrease in pH. When the cells have no supply of nitrogen a still more severe decrease in rate develops with time.

The most striking effects of the nitrogen source are seen in the R.Q.² and in the changes in pH of the cell suspension presented in Table I. Uptake of nitrate is characteristically accompanied by a high R.Q. and an increase in pH; uptake of ammonia, by a lower R.Q. and decrease in pH. Similar changes in pH have been observed by Pratt and Fong (1940). Apparently nitrate is

TABLE I

Variation of the R.Q. with the Nitrogen Source during Glucose Assimilation by Growing Cells
The R.Q.'s were calculated over the time period of 200 to 300 minutes for each experiment.

Experiment	Initial pH		Nitrogen source			
			NO ₃ ⁻	No nitrogen	NH ₄ ⁺	NH ₄ ⁺ + NO ₃ ⁻
1	4.5	R.Q.	1.58	1.31	1.22	—
		Final pH	6.0	4.7	3.1	—
2	6.8	R.Q.	1.66	1.42	1.18	—
		Final pH	7.6	6.9	3.5	—
3	4.5	R.Q.	1.58	—	—	1.16
		Final pH	5.9	—	—	3.5

absorbed in exchange for a hydroxyl ion and ammonium in exchange for a hydrogen ion (or at least this is the net effect). The increase in pH on nitrate to a value of about 6.0 and attendant retention of carbon dioxide as bicarbonate must cause the calculated rate of carbon dioxide evolution (Fig. 1) and the R.Q. (Experiment 1 of Table I) to be somewhat too low.

That the pH itself is not the cause of the difference in R.Q.'s is demonstrated by other experiments at an initial pH of 6.8 (*cf.* Experiment 2 of Table I). Still other experiments by the indirect method, using flasks with different liquid: gas ratios yielded R.Q.'s of 1.57 to 1.61 on nitrate, confirming those obtained by the direct method.

Under no conditions has an R.Q. of 1.0 been observed in the respiration of glucose by growing cells. Even with no available nitrogen source the R.Q. is considerably greater than unity, indicating assimilation of the glucose to

² For convenience the R.Q. and other CO₂/O₂ quotients are cited without inclusion of the negative sign which obtains from the usual conventions of gas exchange.

more reduced cellular materials. Under similar conditions Gaffron (1939) has observed R.Q.'s varying from 1.2 to 2.0 in a number of different algae. Another related observation reported by Spoehr and coworkers (1946) is that *Chlorella* shows a greatly increased fat production during nitrogen deficiency.

If both nitrate and ammonia are provided the algae (Experiment 3 of Table I) the resulting R.Q. and change in pH clearly show that no nitrate reduction occurs when ammonia is present. This observation confirms the conclusion

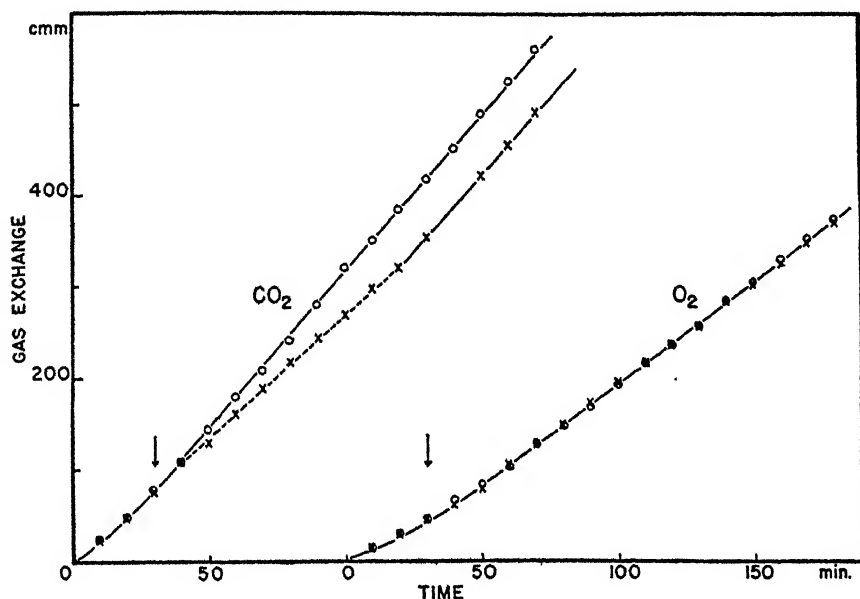


FIG. 2. Gas exchange accompanying assimilation of nitrate and ammonia during dark assimilation of glucose. Experimental details: 26 c.mm. growing cells per flask in Knop's containing nitrate and glucose; 1.0 micromol $(\text{NH}_4)_2\text{SO}_4$ (xxxx) or zero $(\text{NH}_4)_2\text{SO}_4$ (oooo) added at the time indicated by the vertical arrows.

of Pratt and Fong (1940) that when both nitrogen sources are available *Chlorella* utilizes ammonia in preference to nitrate. Since nitrate reduction must result in products of the state of reduction of ammonia, and since no nitrate reduction occurs when ammonia is available, it follows that nitrate is reduced only in a manner intimately related to the requirements of subsequent nitrogenous synthesis.

The suppression of nitrate reduction by ammonia in growing cells submits to further experiments such as that shown in Fig. 2. If 2 micromols of ammonia are added to cells assimilating glucose and nitrate the rate of oxygen uptake is not at all affected. Carbon dioxide output, however, is reduced almost immediately to a lower constant rate which persists until the ammonia has been

utilized; the rate then returns abruptly to a value characteristic of nitrate assimilation. The R.Q. during nitrate assimilation is 1.59, during ammonia assimilation 1.22, in good agreement with the values cited in Table I. Again it is apparent that ammonia suppresses nitrate reduction. Still more striking is the evidence that the higher R.Q. during nitrate reduction results entirely from extra- CO_2 in agreement with comparable observations of Warburg and Negelein.

Nitrate Reduction during Carbon Dioxide Assimilation in Light

Many measurements of the assimilatory quotient (CO_2/O_2) for *Chlorella* in the usual nitrate-containing Knop's solution have yielded values of about 0.9 (cf. Warburg, 1948; Emerson and Lewis, 1941). Invariably the measurements

TABLE II

Variation of the CO_2/O_2 Quotient with the Nitrogen Source at Low Light Intensity

40 f.-c. illumination; 4 per cent CO_2 ; ~ 12 c.mm. cells/flask; Knop's at pH 4.5 with nitrogen source as indicated.

NO_3^-		NH_4^+	$\text{NO}_3^- + \text{NH}_4^+$
0.76	0.68	0.91	0.94
0.68	0.65	0.96	0.93
0.67	0.66	0.91	
0.71	0.60	0.95	
0.70	0.64	0.96	
		0.97	
Average.....	0.68	0.94	0.94

have been made at photosynthesis-saturating light intensities. Under such conditions the cells used here have an A.Q. of 0.88. Light saturation of growth, however, occurs at an intensity considerably below that required for photosynthesis as observed in short time experiments (Myers, 1946). For the present work a light intensity of about 40 f.-c. was chosen in order to approximate the illumination under which the cells had been growing. This intensity may be described as light-limiting for both growth and photosynthesis and affords an oxygen evolution about five times as great as the endogenous oxygen uptake in the dark.

Presented in Table II are a series of measurements of the CO_2/O_2 quotient obtained by the indirect method. Since these were short experiments of about 90 minutes duration the pH (on nitrate) never rose to a value greater than 4.9 and retention of carbon dioxide could be neglected as a close approximation. The actual rates of oxygen and carbon dioxide exchange are omitted since small variations in light intensity between experiments preclude comparisons

on this basis. The quotient of gas exchange, however, is relatively insensitive to small variations in light intensity.

In light metabolism, as during glucose assimilation in the dark, nitrate reduction is apparent as a marked effect on the gas exchange quotient. The low quotient of 0.68 on nitrate has been confirmed by mass culture experiments at low light intensity which yield a quotient of 0.71 (Myers and Johnston, 1949). Again the quotients obtained with both nitrate and ammonia present are identical with those obtained with ammonia alone, indicating that nitrate reduction during photosynthesis is linked to metabolism and is not an incidental process. The important problem of the effect of light intensity on the quotient during nitrate assimilation will be treated in the following paper.

TABLE III

The Change in Gas Exchange Caused by Addition of Ammonium Sulfate after a Period of Nitrate Utilization

Neon illumination equivalent to 40 f.-c. of tungsten illumination; 4 per cent CO₂; ~ 24 c.mm. cells/flask; original solution Knop's at pH 4.5; gas exchange computed in c.mm./hour.

Experiment	O ₂ evolution			CO ₂ uptake		
	NO ₃ ⁻	NH ₄ ⁺	Change	NO ₃ ⁻	NH ₄ ⁺	Change
1	101	97	-4.0	69	98	+29
2	96.5	97	+0.5	77	104	+27
3	90	89.5	-0.5	63.5	90.5	+27
Average change.....			-1.0			+28

The low quotient on nitrate may be related to nitrate reduction. Unfortunately the experiments reported in Table II do not allow decision whether the low quotient accompanying nitrate reduction is the result of increased oxygen production or decreased carbon dioxide uptake. This problem has been attacked by addition of ammonium salt after a period of nitrate reduction.

Duplicate flasks with a circular flat bottom 10 cm.² in area and a sidearm were used with different liquid:gas ratios. About 5 mg. of dry ammonium sulfate were placed in the sidearm in lieu of a solution in order to eliminate any question of gas exchange equilibrium between cell suspension and fluid in the sidearm. A negligible volume change is introduced on addition of so small a quantity of solute. Since it is necessary to determine the gas exchange before and after addition of the ammonium salt the duplicate flasks cannot be exchanged in position in any one experiment. Two flask positions over the neon grid were selected at which the light intensities matched to within 1 per cent and in successive experiments the two flasks were used alternately in the two positions.

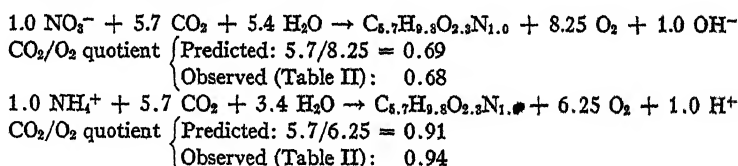
The results of three representative experiments are summarized in Table III. The quotients of gas exchange are here not very reliable since the dupli-

cate flasks required for each determination did not receive precisely the same illumination. The procedure does allow comparison of the changes in rates of oxygen and carbon dioxide exchange accompanying the transition from nitrate to ammonia utilization. The change in rate of oxygen evolution is small and perhaps negligible; the increased rate of carbon dioxide uptake is consistently large. The low quotient of nitrate reduction during photosynthetic metabolism is principally due, therefore, to a depression in carbon dioxide uptake. Such a result is to be expected on theoretical grounds. In the over-all metabolism nitrate reduction and carbon dioxide reduction are competing endergonic processes. Under light-limiting intensities the photochemical process limits the level of total metabolic energy expenditure.

Considerations from Cell Analysis

During photosynthetic growth of *Chlorella* in mass cultures about 95 per cent of the carbon dioxide and nitrate taken up can be recovered as cellular carbon and nitrogen (Myers and Johnston, 1949). As a close approximation, therefore, new cell material and oxygen are the only products of photosynthetic metabolism.

Elementary analysis of *Chlorella*, as grown for the present experiments, yields 53 per cent C, 7.5 per cent H, 28.5 per cent O, and 10.8 per cent N on an ash-free, dry-weight basis. On dividing by the appropriate atomic weights these percentages can be converted to the expression $C_{5.7}H_{9.8}O_{2.3}N_{1.0}$. By such a procedure (*cf.* Tamiya, 1932) it becomes possible to write balanced equations for over-all metabolism and thus predict the gas exchange required for the appropriate nitrogen source. Thus



If the two equations are to be compared on a rate basis, then all of the integers of the equation for nitrate utilization should be multiplied by the factor 6.25/8.25 to allow equal rates of oxygen production as demanded by the data of Table III. The equations are written, however, only with the intent of demonstrating the relative proportions of the various reactants and products of over-all metabolism. It is seen that in an organism such as *Chlorella* the over-all metabolism can be predicted equally well from cell analysis or from measurements of the gas exchange. Similar equations could be written for glucose assimilation in the dark but would require additional information on the fraction of the glucose assimilated by growing cells.

DISCUSSION

The characteristics of nitrate reduction in *Chlorella* under growth conditions may be compared with those observed by Warburg and Negelein (1920) in their highly acid nitrate mixture. Growing cells do not excrete ammonia; they reduce nitrate only at a rate compatible with further nitrogenous synthesis. This is borne out also by experiments with nitrogen-deficient cells (to be reported elsewhere) in which the rate of nitrate reduction may be very much increased. The limiting factor for nitrate reduction cannot be ascribed to the rate of entrance of nitrate into the cells. Warburg and Negelein attributed the success of their acid nitrate mixture to its high concentration of neutral HNO_3 molecules to which the cells are presumably more permeable. At the same time their acid medium, aside from its possible internal effects on the cells, also provided conditions more favorable for ammonia excretion. A change of pH from 5.0 to 2.0 decreases the external concentration of undissociated NH_4OH a thousandfold. If the internal pH of the cell remains reasonably constant, and if the only membrane-penetrating species of ammonia is the undissociated form, then at the lower pH more favorable conditions for ammonia excretion will obtain.

The observed effect of the nitrate in decreasing the carbon dioxide uptake under the light-limiting illumination used here (Table III) is entirely compatible with the extra- O_2 reported for the nitrate mixture. Warburg and Negelein did, in fact, explain their extra- O_2 as a primary extra- CO_2 produced by equation (1) and then converted photosynthetically to extra- O_2 under their conditions of light saturation and carbon dioxide limitation.

The present data offer no comparison between the actual rates of nitrate reduction in light and in darkness and therefore allow no explanation of the accelerating effect of light observed by Warburg and Negelein. The whole problem of the mechanism of nitrate reduction in light and its relation to photosynthesis will be treated in subsequent work.

Comparison of the present work on *Chlorella* may also be made to the work of Burström (1942, 1943) on nitrate assimilation in wheat leaves. Young excised wheat leaves, when illuminated, show an uptake of carbon dioxide, an increase in sugar content, and a decrease in nitrate content. Over a considerable range of light intensity only part of the carbon assimilated is recovered as sugars; the remainder is quantitatively related to the nitrate consumed. In darkness the carbon of the sugars consumed is quantitatively recovered as carbon dioxide and no nitrate is reduced. This latter characteristic, which marks an important difference between the wheat leaf and *Chlorella*, allowed Burström to postulate that nitrate reduction is intimately linked to the photosynthetic process.

Finally, it may be pointed out that a tool of considerable possible usefulness

has been developed for the study of nitrogen metabolism. The high nitrogen content of *Chlorella*, the differential effect of nitrate and ammonia on its gas exchange, and its characteristic of suppression of nitrate reduction by ammonia offer particular advantages in studies on the mechanism of nitrogen assimilation.

SUMMARY

1. Nitrate reduction and assimilation have been studied in *Chlorella pyrenoidosa* under growth conditions by observing effects on the CO_2/O_2 gas exchange quotient.

2. During assimilation of glucose in the dark, nitrate reduction is noted as an increase in the R.Q. to about 1.6 caused by an increased rate of carbon dioxide production.

3. During photosynthesis at low light intensity nitrate reduction is evidenced by a reduction in the CO_2/O_2 quotient to about 0.7 caused by a decreased rate of carbon dioxide uptake.

4. *Chlorella* will assimilate nitrogen from either nitrate or ammonia. When both sources are supplied, only ammonia is utilized and no nitrate reduction occurs. It is inferred that under the usual conditions of growth nitrate is reduced only at a rate required for subsequent cellular syntheses. The effect of nitrate reduction on the CO_2/O_2 quotient therefore provides a measure of the relative rate of nitrogen assimilation.

5. Over-all photosynthetic metabolism may be described from elementary analysis of the cells since excretory products are negligible. The gas exchange predicted in this way is in good agreement with the observed CO_2/O_2 quotients.

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METABOLIC CONDITIONS IN CHLORELLA*

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The variability of metabolism observed quite generally in microorganisms has never been systematically studied in the green algae. The frequent use of forms such as *Chlorella pyrenoidosa* in the study of photosynthesis has led to common use of purposely standardized culture conditions. This practice has been pushed toward its limit in the development of a continuous culture apparatus (Myers and Clark, 1944) which provides uniform experimental material day after day. At the same time the continuous culture method also affords a basis for the study of variability in metabolism imposed by various conditions.

The quotient of gas exchange has long been recognized as a practical tool in metabolic studies. Its usefulness in *Chlorella* has been extended by the work of the preceding paper (Cramer and Myers, 1948 b) which showed that the effect of nitrate reduction on the gas exchange quotient provides an index of the rate of nitrogenous synthesis. This finding is now applied to cells subjected to such conditions as might be expected to affect their metabolic activities.

The present study began in an attempt to explain the effect of light intensity on the CO_2/O_2 quotient noted in the preceding paper. It has been extended to include consideration of the changes in over-all metabolism induced by starvation, high light intensity, and nitrogen deficiency in comparison with the metabolism of growing cells. The general experimental methods and the over-all carbon and nitrogen metabolism in growing cells have been described in the preceding paper. It should be emphasized that the term *growing cells*, as used herein, specifically describes a standard preparation; *i.e.*, cells cultured photosynthetically under an illumination light-limiting for both growth and photosynthesis. The merit of this particular choice of reference will be justified in the subsequent discussion.

Starvation

Growing cells may be starved aerobically by shaking a suspension in the dark. Starvation results in a gradual decrease in capacity for photosynthesis, a disappearance of starch, and a marked decrease in endogenous respiration which approaches a constant rate with an R.Q. of 1.0 (Cramer and Myers, 1948 a). Table I describes the time course of the R.Q. after addition of glucose to growing and starved cells.

* Supported by a grant from the University of Texas Research Institute.

Initial R.Q.'s of starved cells in nitrate are similar to those of growing cells in ammonia, indicating complete absence of nitrate assimilation in starved cells, although it is known that carbohydrate assimilation is taking place during this time (Myers, 1947).¹ Only after a considerable period of assimilation do starved cells exhibit the high R.Q. resulting from nitrate reduction that is characteristic of growing cells. This is borne out also by the final pH values of the cell suspensions. Nitrate uptake is accompanied by an increase in pH and the higher final pH values are related to the longer periods of high R.Q. and nitrate assimilation. The later R.Q. values for growing cells are probably somewhat low due to the high final pH and retention of carbon dioxide as bicarbonate.

TABLE I

Variation of R.Q. and pH during Glucose Assimilation in Starved Cells
In Knop's solution with nitrate as the nitrogen source. Original pH = 4.45.

Time interval	R.Q.		
	Growing	Starved 1 day	Starved 3 days
<i>min.</i>			
0-50	1.46	1.10	1.22
50-100	1.55	1.18	1.15
100-150	1.57	1.53	1.26
150-200	1.52	1.58	1.35
200-250	1.57	1.65	1.47
250-300	—	—	1.65
Final pH.....	5.9	5.6	4.5

Effects of the nitrogen source and light intensity on the CO_2/O_2 quotient in growing and starved cells are presented in Table II. The low light intensity of 40 foot-candles was chosen as being light-limiting for both growth and photosynthesis and approximately the same as the effective light intensity of culture; the high light intensity of 600 f.-c. is light-saturating for both growth and photosynthesis. Each of the data describes the CO_2/O_2 quotient during the period of 30 to 90 minutes after the beginning of illumination. At both light intensities of measurement the CO_2/O_2 quotients of starved cells on nitrate approach a value of 1.0 and approach still more closely the quotients found in growing cells on ammonia where no nitrate reduction is taking place. Evidently the metabolism of starved cells requires a high C/N assimilation ratio.

¹ Attention is called to an author's error in Table III of the paper cited where the R.Q. values for glucose and acetic acid were reversed. Observed values of the R.Q. on acetic acid were 0.99 to 1.04; on glucose, 1.09 to 1.16. The latter values are in agreement with the initial R.Q.'s observed here.

High Light Intensity

Table II also contains data bearing on the metabolism at high light intensity. Growing cells studied at high light intensity in nitrate media show a CO_2/O_2 quotient of 0.88 in agreement with the often cited value of 0.9. The differential in the quotient between nitrate and ammonia (0.88 *vs.* 0.95) is smaller than the differential observed at low light intensity (0.68 *vs.* 0.94).

TABLE II

Variation of the CO_2/O_2 Quotient with Nitrogen Source and Light Intensity

Knop's at pH 4.5 with nitrogen source as indicated; ~ 12 c.mm. cells/flask; 4 per cent CO_2 .

Nitrogen source	Measured at 40 f.-c.			Measured at 600 f.-c.		
	Growing	Starved 1 day	Starved 3 days	Growing	Starved 1 day	Starved 3 days
NO_3^-	0.76	0.82	0.89	0.86	0.90	0.95
	0.68	0.81	0.88	0.87	0.93	0.95
	0.67	0.87	0.96	0.88	0.90	0.95
	0.71	0.86	0.91	0.86	0.91	0.98
	0.70	0.85	0.92	0.87		
	0.68	0.89		0.88		
	0.65			0.90		
	0.66			0.90		
	0.60					
	0.64					
Average.....	0.68	0.85	0.91	0.88	0.91	0.96
NH_4^+	0.91			0.94		
	0.96			0.96		
	0.91			0.94		
	0.95			0.97		
	0.96			0.92		
	0.97			0.95		
Average.....	0.94			0.95		

At high intensities there must occur proportionately less nitrogen synthesis and proportionately greater carbohydrate synthesis.

After 3 hours' exposure to an intensity of 600 f.-c. and transfer to fresh Knop's solution (with nitrate) the CO_2/O_2 quotients observed at low light intensity were 0.37 and 0.44. Such experiments led to a study of the CO_2/O_2 quotient in cells which had been grown at high light intensity (300 f.-c.) in the continuous culture apparatus. Comparison of cells grown at high and low light intensities is presented in Table III. Cells cultured at high light intensity show a greater rate of respiration and a higher R.Q. indicative of considerable

accompanying nitrate reduction. Under low light intensity of measurement the same cells show a very low CO_2/O_2 quotient again indicating rapid nitrate reduction. All the data indicate that at high light intensities there occurs an increase in ratio of C/N assimilation. On return to low light intensities the

TABLE III

Effect of Light Intensity of Culture on the Subsequent Gas Exchange

Medium Knop's solution (with nitrate) at pH 4.5. Rates of gas exchange in c.mm./hr./c.mm. cells.

	Cells grown at high light intensity (300 f.-c.)			Cells grown at low light intensity (45 f.-c.)		
	O_2	CO_2	CO_2/O_2	O_2	CO_2	CO_2/O_2
Low light (45 f.-c.)	3.08	-0.97	0.32	5.35	-3.19	0.60
Darkness	-2.05	3.23	1.58	-1.47	2.07	1.41

TABLE IV

Gas Exchange of Normal and Nitrogen-Deficient Cells

Measurements made before and after exposure to high light intensity in Knop's solution minus nitrate. Rates of gas exchange in c.mm./hr./c.mm. cells (referred to the cell volume before exposure).

Experiment	Studied at	Before exposure			Time of exposure	After exposure		
		O ₂	CO ₂	CO ₂ /O ₂		O ₂	CO ₂	CO ₂ /O ₂
1	Low light (40 f.-c.) Darkness	Growing cells in Knop's (+NO ₃ ⁻)			4.5	Nitrogen-deficient cells in Knop's (+NO ₃ ⁻)		
		4.57	-3.17	0.69		3.92	-0.65	0.17
		-1.68	2.23	1.33		-3.09	4.85	1.57
2	High light (600 f.-c.) Darkness	42.5	-36.5	0.86	3.0	39.0	-28.7	0.74
		-1.37	1.79	1.31		-2.04	3.24	1.59
3	High light (600 f.-c.) Darkness				4.0	Nitrogen-deficient cells in Knop's minus NO ₃ ⁻		
						31.6	-31.4	0.99
						-1.38	1.38	1.0

C/N assimilation ratio decreases to a value still lower than that characteristic of cells always grown under low light intensity.

Nitrogen Deficiency

Effects of the C/N assimilation ratio may also be expected as a result of limited nitrogen supply. Nitrogen-deficient cell preparations could be ob-

tained by transferring growing cells to a Knop's solution with the usual KNO_3 replaced by K_2SO_4 and illuminating at a high intensity (~ 300 f.c.) under 4 percent carbon dioxide. Three typical experiments are detailed in Table IV. When the nitrogen-deficient cells are taken up in fresh nitrogen deficient media (Experiment 3) the CO_2/O_2 quotients of 1.0 indicate a com-

TABLE V
Summary of Four Metabolic Conditions in *Chlorella*

Metabolic condition	CO_2/O_2 quotient with NO_3^-		C/N ratio of assimilates	C/N ratio of cells (inferred)
	In low light	In high light		
Growing (at low light intensity).....	0.7	0.9	Normal	Normal
Starved.....	0.9	1.0	>Normal	<Normal
High light exposed.....	~ 0.4	—	<Normal	>Normal
Nitrogen-deficient.....	~ 0.2	0.7	<Normal	>Normal

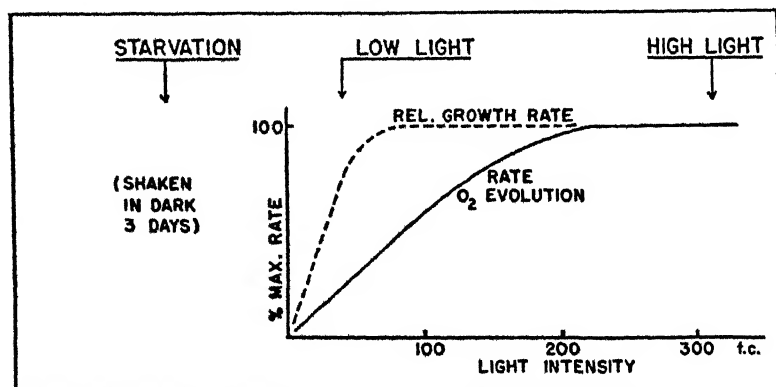


FIG. 1. Graphical description of conditions which induce three different types of metabolism in *Chlorella*. Curves for relative growth rate and rate of oxygen evolution are idealized from experimental curves (Myers, 1946).

pletely carbohydrate metabolism.² On return to nitrate-containing media (Experiments 1 and 2) the nitrogen-deficient cells exhibit a CO_2/O_2 quotient depressed markedly at low light intensities and significantly even at high intensities. The return of a nitrogen supply results in a C/N assimilation ratio far below the normal ratio of growing cells.

² Note, however, that these are short time experiments. It is likely that on longer exposure in nitrogen-deficient media considerable lipid materials may be produced (*cf.* Spoehr and coworkers, 1946).

DISCUSSION

Characteristics of the four types of cells studied are summarized in Table V and may be considered with reference to Fig. 1. One is forced to select as a *normal* or *reference* condition that which occurs during growth at low light intensity. Here the rate of carbon assimilation limits the rate of growth and metabolism is not "growth bound." Assimilated carbon distributes itself freely between the nitrogenous, carbohydrate, and lipid cell constituents. As the rate of carbon assimilation increases under increasing light intensities a point is reached at which some factor, very likely the rate of nitrogen assimilation, becomes growth-limiting. Further increase in light intensity to a saturating intensity for photosynthesis now results in a greater and greater proportion of carbohydrate synthesis. On return to low light intensity, however, the cells which are glutted with carbohydrate now show a rapid nitrate assimilation with remarkably low CO_2/O_2 quotient.

Nitrogen deficiency, as high light intensity, leads to a predominantly carbohydrate metabolism. On return to a nitrate supply, nitrogen-deficient cells show evidence of very rapid nitrate assimilation in their very low CO_2/O_2 quotients. Starvation, on the other hand, decreases the proportionate amount of carbohydrate so that starved cells at either high or low light intensity or in the dark show quotients approaching those of carbohydrate synthesis alone. Starved cells can again become growing cells only after restoring the C/N balance characteristic of growing cells.

The variable metabolic pattern in *Chlorella* has practical importance with regard to studies on photosynthesis. The quantum yield must be measured at low intensities where the CO_2/O_2 quotient is particularly sensitive to metabolic conditions. In reviewing work on this problem it is difficult to decide whether the algae used were growing or starved in the sense used here. Attention may be called to the wide variation in the CO_2/O_2 quotient observed by Manning, Stauffer, Duggar, and Daniels (1938) in their quantum efficiency studies on *Chlorella* in nitrate-containing media. The CO_2/O_2 quotient varied so widely that the average value of 1.02 cited by Rabinowitch (1945) is of doubtful significance. It now appears possible that these variations are explainable in terms of varying rates of nitrate reduction in response to the metabolic conditions of the cells used.

The present observations also have bearing on studies of *Chlorella* at high light intensities. In the usual short time manometric experiments at photosynthesis-saturating light intensity a constant rate of gas exchange may continue for an hour or more. Strictly steady state conditions do not obtain, however, since the cellular composition is shifting in the direction of increasing carbon content and the shift cannot continue indefinitely at a constant rate. Additional information on the effects of high light intensity has been sought in mass culture experiments and will be reported elsewhere.

It is instructive to apply to *Chlorella* the same considerations of nutritional economy which Foster (1947) has proposed for the interpretation of mold metabolism. Under frugal nutritional conditions, as experienced in their natural habitat, the molds are highly efficient in converting substrates to cell materials. When provided with high concentrations of carbohydrate or when limited in nitrogen supply their metabolism becomes deranged to an *overflow* metabolism with the conversion of large amounts of substrate to storage or excretory products. Nutritional efficiency in *Chlorella pyrenoidosa* is likewise geared to a marginal economy; it will grow at a light intensity of less than 10 f.-c. and reaches a maximum growth rate at less than 100 f.-c. (Myers, 1946). High light intensity must provide here the same conditions of overabundant metabolic substrate (reduced products of carbon dioxide) that high carbohydrate concentrations provide for molds, but the lack of extensive excretion limits the overflow products to storage carbohydrate and lipid. From such considerations the metabolism of *Chlorella* in high light intensity may be interpreted as an abnormal or overflow metabolism.

SUMMARY

1. The effect of nitrate reduction and assimilation on the CO_2/O_2 quotient of gas exchange has been used as an index of the relative rates of carbon and nitrogen assimilation in *Chlorella pyrenoidosa*. Changes in over-all metabolism induced by starvation, high light intensity, and nitrogen deficiency have been studied in comparison with the metabolism of cells growing at light-limiting intensities.

2. Starvation, which results in depletion of carbohydrate reserves, gives rise to a high CO_2/O_2 quotient (~ 0.9) during photosynthesis and, therefore, a high C/N assimilation ratio. Starved cells apparently restore their normal C/N ratio before becoming growing cells.

3. Under photosynthesis-saturating light intensities cells show the high CO_2/O_2 quotient (0.9) indicative of a high C/N assimilation ratio. Return to low light intensities is followed by the abnormally low CO_2/O_2 quotient (~ 0.4) of a low C/N assimilation ratio. High light intensity apparently gives rise to a condition of a limiting rate of nitrogen assimilation and to an overflow metabolism analogous to that found in other microorganisms.

4. Nitrogen deficiency leads to a completely carbohydrate metabolism in short time experiments and makes still more pronounced the effects characteristic of high light intensity alone.

5. Considerations of nutritional economy sustain the experimental evidence in establishing the metabolism of cells growing under light-limiting intensities as the normal or reference metabolic condition in *Chlorella*.

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STUDIES ON THE PHYSIOLOGICAL EFFECTS OF NON-POLAR-POLAR ORGANIC ELECTROLYTES

II. THE INFLUENCE OF DETERGENTS UPON THE POTENTIOMETRIC REACTION AND THE CONTRACTILITY OF NERVE AND MUSCLE

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INTRODUCTION

In his first study on the influence of inorganic neutral alkali salts upon the resting potential of frog muscle Höber (1) came to the conclusion that the results can be interpreted by assuming that they are called forth by alterations of the colloidal constituents of the plasma membrane of the muscle fibers and that the same alterations, swelling or shrinking, appear during the excitatory process, —in other words, during the action of the potential wave. Later, the same interpretation was applied by Netter (2) in a corresponding study of the effects of neutral inorganic alkali salts on frog nerve which is the most appropriate organ to respond to an ionic imbalance with an electric reaction. Subsequently, these experiments with inorganic salts were extended to observations comparing on one hand the myelinated frog nerves with unmyelinated ones of crabs; on the other hand, inorganic ions were compared with organic ions in order to test the properties of the surface membrane as correlated with lipoid solubility, surface activity, porosity, etc. (see especially Wilbrandt (3), Höber, Andersch, Höber, and Nebel (4). See further Shanes and Brown (5)). In connection with these latter investigations recently we have included in our study a special group of organic electrolytes, the *detergents*. This term refers here to the large group of organic strong electrolytes, especially the sodium salts of strong acids, the anions of which have a non-polar-polar molecular configuration, the polar hydrophilic character of which ordinarily is due to a sulfate or sulfonate group on one side of the anion, the non-polar (organophilic, hydrophobic) character to a greater or smaller number of alkyl groups on the other side of the oblong structure. A first series of observations about the influence of non-polar-polar detergents on muscle potentials has been described previously (see Höber (6)). A parallel study on frog sciatics follows in this paper although this object is by far not so favorable as frog muscle. Nerve fibers of frog are wrapped in layers of dense connective tissue, which may be split by a quartz needle (Gerard), but not without damaging by this

procedure many of the enclosed more or less isolated fibers. Furthermore, the myelin sheath of the fibers of a frog's sciatic probably fails to be penetrated by the neutral strong detergent electrolytes, so that one may be confronted with the fact that the detergent salts do not come in contact with the plasma membrane (or neurilemma) except at the Ranvier nodes (see Kato (7)). For these reasons future studies will include investigations on unmyelinated nerves.

Methods

The excised nerves were placed in a moist chamber as described by Netter (2). This chamber can easily be enlarged for the use of four nerves, the resting potentials of which are measured alternately (see also Wilbrandt (3), Shanes (5)).

RESULTS

The results are shown in Table I, which strikingly resembles Table I in our first paper (p. 392). Again, the + and - signs indicate the direction of the resting potentials, referred to the initial potential, observed under the influence of Ringer solution.

In other words, a + sign indicates a potential which, arising under the local influence of one of the active (detergent) substances, is directed in the outside branch of the electric circuit from the point of local influence towards the unaltered surface, whereas a - sign indicates a potential comparable to an injury potential; *i.e.*, the current flows outside the fibers from the intact surface to the altered surface.¹

The numerical values of the potentials (in millivolts) appearing under the influence of the same solution vary from one experiment to the other, whereas the direction of the potential fall is independent of the concentration.²

Taking this into account, it is obvious that the behavior of the muscles, as shown in Table I of the first paper,¹ is fairly well duplicated by Table I of this paper regarding nerve.³

However, it seems necessary to interpret our results somewhat more in detail than in the report on muscle in the first paper. The effect of the various

¹ + → - means a potential changing during the experiment spontaneously from a positive toward a negative direction. 0 indicates no effect.

² Comparing the amount of potential appearing in our former experiments with muscle, the shifts in millivolts, which are brought about on nerves, are considerably smaller.

³ But again attention has to be paid to the fact that as in the former paper (p. 393) our results are dealt with only in a highly abbreviated form, disregarding individual, seasonal, environmental, and other influences, for the reason explained in the first paper.

detergents is different with regard to the length of the alkyl chains, which regulates the adsorption affinity; also a difference arises in regard to the reversibility or eventual irreversibility of their attachment, especially in correlation to the concentration applied. This adsorption brings about an

TABLE I
Resting Potentials of Frog Nerves
Molar Concentrations Producing Either Negative or Positive Resting Potentials

Molar concentrations.....	1/1000	1/500	1/300	1/250	1/200	1/100	1/50	1/40	1/30	1/20	1/15	1/13.3	1/12.5	1/10
<i>Alkylbenzene sulfonates</i>														
9 C:nonyl		0			+→-	-	-							
8 C:octyl			+→-			-								
6 C:hexyl					+	+	+→-		-					
4 C:butyl											+	+	+→-	
3 C:isopropyl										0			+	+
2 C:ethyl														+
2 C:xylene														+
1 C:toluene														+
0 C:benzene														
<i>Alkyl sulfates</i>														
12 C:dodecyl	+	-												
10 C:decyl		0			+									
8 C:octyl		0		-										
7 C:diisopropylcarbinol							+							
6 C:2-ethylbutyl						0				+				+
4 C:isobutyl										+				+
<i>Dialkyl sulfosuccinates</i>														
16 C:dioctyl OT	-													
12 C:dihexyl MA				+		-								
10 C:diamyl AY		0		+		+→-				-				
8 C:dibutyl 1B		0		0		0		0	0	+→-				-

orientation of the non-polar-polar anion with its non-polar half directed to the membrane, its polar half directed towards the aqueous phase in the surroundings. The result of this attachment is a negative charge of the membrane substance or, better, an increase of the natural negative charge of the substance (its negative *electrokinetic potential*) which is independent of the natural *thermodynamic positive potential*. This latter one is based upon the normal selective permeability of the membrane to K ions accumulated in the interior of the fiber and due to the small size of K ion in contrast to the large size of the other alkali cations, particularly Na.

Thus it follows that if the membrane is locally exposed to a certain concentration of an appropriate detergent anion, the thermodynamic membrane potential at that spot is changed, *i.e.* first increased (in other words, more positive), because the natural negative charge of the membrane substance will become more negative, so that the thermodynamic potential with K outside rises locally; a *reversed current* appears.

But with higher concentrations of detergent anion not only the charge, but also the architecture of the membrane substance will be altered. Due to their adsorption, the detergent anions intrude into the array of micelles of the membrane substance and enlarge the intermicellar distances so that the structure of the thermodynamically active membrane is loosened up; consequently it becomes more permeable even to Na and other inorganic cations having a size greater than the size of K and also permeable to inorganic anions. The local positive thermodynamic potential falls off, eventually to zero, the reversed resting potential disappears and turns in the direction of the normal injury current, issuing from a normal surface spot and directed to the injured spot. The final result of the loosening effect of the detergent adsorption seems to be a breakdown, or a hole; possibly the membrane is torn to pieces, and if so the effect may become irreversible. It is understandable that this is most probably a result of detergents in higher concentration and with strongly adsorbable long alkyl chains (cytolytic dispersing effect; see the first paper).

Are these alternative electrometric actions of the detergents indicative of other physiological symptoms? It seems promising to correlate these electromotive effects with the most prominent electric phenomenon following excitation, the *action potential wave*, or the propagated disturbance following stimulation. This is a wave of depolarization of the plasma membrane issuing from the stimulated spot and immediately succeeded by a repolarization, while a more distant spot undergoes depolarization. This sequence, according to Hermann (1876) and others is interpreted by the *Stroemchentheorie*, which has been worked out more in detail by many other authors in the "core conductor theory" (Cremer (8), Ebbecke (9), Lillie (10), Labes and Zain (11), and others), particularly by using the so called "*membrane core conductor*" as pictured in Fig. 1.

In this model the propagated diphasic action potential wave is reproduced as follows: *NN* is a part of a nerve fiber. *pppp* is a tube made of a hydrophilic colloid and representative of the plasma membrane or the neurilemma; the tube is filled with a neutral solution of potassium phosphate as axoplasm and imbedded into a solution of NaCl by soaking the gauze which wraps the colloidal tube in it.

According to the studies of Ebbecke and Labes, it is chiefly shown that this model acts in the following manner: Due to its greater permeability to K and Cl, compared to permeability to Na and phosphate, the outside of the colloidal

tube is positive, the inside negative. Upon local (mechanical, chemical, or electrical) stimulation, indicated in Fig. 1 by the shaded region, the structure of such a tube can be softened so much as to abolish the membrane potential; the membrane becomes depolarized. The result is that in the neighborhood of the stimulated area circuit currents appear, which *below* the stimulated area drive K and Cl into the tube and make its wall undergo a transitory dispersion, whereas *above* it, inside the stimulated area Na and HPO_4 are shifted into the tube and make it tighter; *i.e.*, they repolarize it.

In this way the just depolarized part of the tube must initiate further extension of the circuit formation (as especially shown by experiments of Labes and Zain (11)).

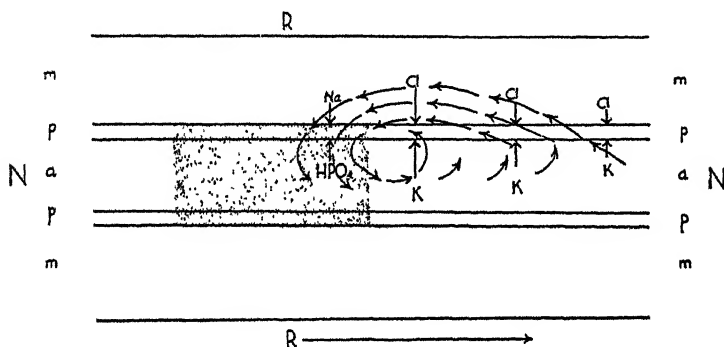


FIG. 1. Model of a membrane core conductor.

Now, returning to our previously mentioned experimental results on the influence of the various detergents upon the electromotive reaction of muscle and nerve, the following experiments on muscle have been carried out: A frog's sartorius is suspended in Ringer's solution and is stimulated rhythmically by maximal electric shocks exhibited in the manner shown in Fig. 2.

With this set-up, upon addition of appropriate amounts of various detergents to the Ringer solution the results shown in Table II were obtained.

Each of the Table II *a*, *b*, and *c* presents for each of our three main groups of detergents a remarkable correlation between the potentiometric reaction and the contractility of the frog muscle. The *potentiometric reaction* is, as has been shown before, either a positive (reversed) potential or a negative (injury) potential dependent upon the concentration and the length of the alkyl chain of the compound present. The *contractility* varies in a corresponding fashion being either increased or decreased, and the factual existence of such a correlation between potential changes and contractility is especially secured by the statements given in Table II. This means that for each group the molar concentrations, which have been found to be equally efficient in bringing about an increase or decrease of potential or of contraction, are the same. In other

words, *the changes of electromotoric and of mechanical reactions are intrinsically connected.*

This result is directly understandable with regard to the long chain compounds, since both injury potential and loss of contractility may be conceived as being due to the loosening of the plasma membrane. The further result, however, that there is a correlation between the appearance of a reversed po-

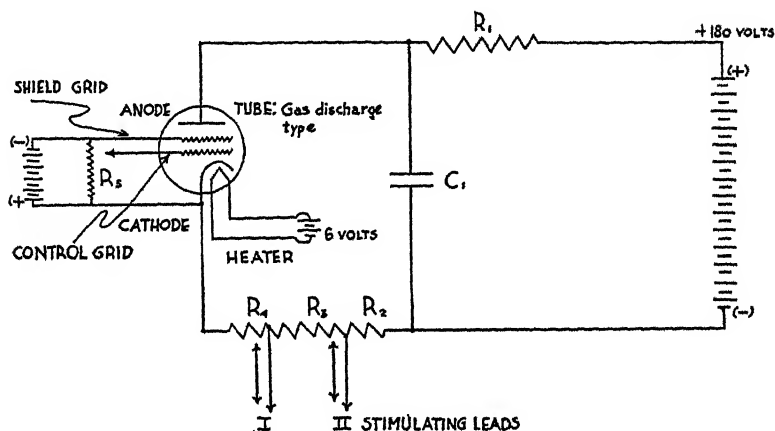


FIG. 2. Set-up for stimulating muscles by rhythmic condenser discharges (J. E. Lilly). A condenser C_1 of 2 to 8 microfarads capacity is charged by a 180 volt battery through the resistor R_1 (0.5 to 2.0 megohms). This circuit is connected with another circuit containing a gas tube (discharge type), through which C_1 can discharge. After the cathode of the tube has been heated enough, at a certain critical voltage the tube suddenly conducts electricity, where previously it did not. The current passes the resistors R_5 , R_3 , R_4 . The critical voltage level, at which the tube discharges the condenser, is controlled by the grid bias voltage on the control grid, which is set by means of R_5 . I and II are the stimulating leads, providing repetitive stimuli at certain intervals (about 10 to 20 per minute).

tential and a rise of contractility may not be simply anticipated, but possibly it can be explained on the basis of the following considerations.

Previously (p. 114) it has been assumed that a reversed potential can be thought of as being primarily indicative of the loosening effect of the intruding detergent anions, but nothing else. A real breakdown beyond loosening would be incompatible with the propagation of the excitation wave which implies the existence of the small circuits, in other words, the *self-repair* of the primarily stimulated spot; and this concept would be in agreement with the well known observations of Cole and Curtis (12), that an excitation wave, running down a nerve fiber (squid), is accompanied by a well reversible increase of ion permeability. But this does not include, as formerly was often suggested, a real

what is more important, through the compounds with *short* alkyl chains in appropriate concentrations, both the thermodynamic and electrokinetic potentials of the membrane can be influenced in such a way (see p. 113) that a reversed potential first arises, but that with more detergent added it turns through zero to the opposite direction, to that of an injury potential.

Let us take at first into consideration the appearance of a positivity. This is connected with a reversible rise of contractility. It leads one to the assumption

effect of the addition, but there becomes evident another symptom of the detergent effect, an alteration of the relaxation curve.

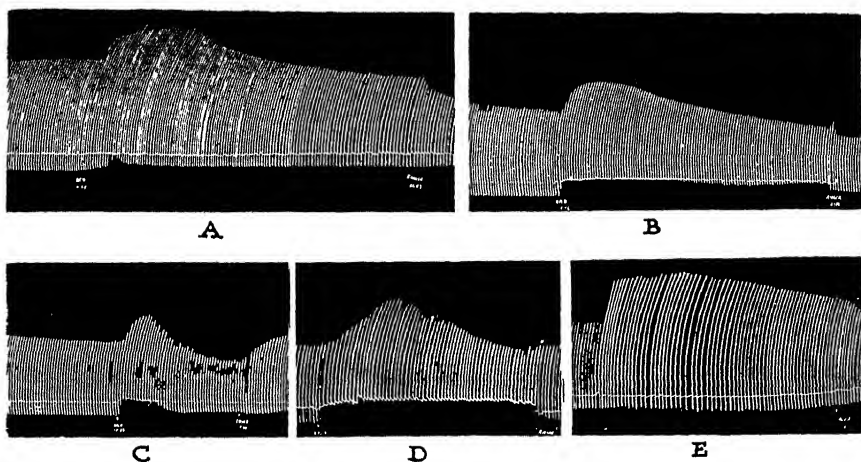


FIG. 3. Effect of detergent solutions upon rhythmical maximal stimulation of frog sartorius muscle. Average interval between stimuli 10 to 12 seconds. *A*, isobutyl sodium sulfate isotonic + K + Ca 3 parts, Ringer 1 part. *B*, 2-ethylbutyl sodium isotonic + K + Ca. *C*, *p*-xylene sodium sulfonate isotonic + K + Ca. *D*, sodium sulfate 0.073 molar (isotonic). *E*, NaSCN isotonic, 1 part + Ringer 3 parts.

Ordinarily this curve swings below the base line of the contraction curve as shown in Fig. 3 *A*. But with certain compounds this swing is stopped immediately after their addition, the stop lasts throughout their presence and disappears promptly after their removal (Fig. 3 *B*). Furthermore, there are closely related compounds, which either fail to produce the "swing stop" or allow it to disappear after some time (Fig. 3 *C*); recording the contraction on a high speed kymograph often brings to evidence irregularities in the relaxation curve or its ending in contracture. Very significant on one hand is the swing stop after applying to the muscle divalent inorganic or organic ions (Fig. 3 *D*), the lack of any swing stop on the other hand after raising the contraction height by univalent inorganic ions, J., NO₃, SCN (Fig. 3 *E*). Probably, in order to interpret these various factors in altering the swing phenomenon, a number of physicochemical reactions, adsorption, swelling, shrinking of the contractile substance, must be considered.

breakdown.⁴ Rather, we come to a conclusion which reaches beyond the results arrived at in Paper I. Detergents are substances which affect the surface membrane of muscle and nerve, as evidenced by the resting potentials as analogues of the excitatory processes. Detergents with *long* alkyl chains are

TABLE II (a-c)

Correlations between the Potentiometric Reactions of the Resting Frog Muscle toward Anionic Detergents and the Response of the Muscle to the Rhythmic Stimulation by Condenser Discharges

	Muscle potential raised (+) or diminished (−) by adding the following molar concentrations					Contraction increased (+) or decreased (−) by adding the following molar concentrations		
(a) <i>Alkyl benzene sulfonates</i>								
10 C:decyl	1/500−	1/1000−	1/2000−	1/4000−		1/150−	1/300±	1/400−
8 C:octyl	1/100−	1/300±	1/400−	1/500−	1/2000±	1/40−	1/100−	1/300− 1/100−
4 C:butyl	1/12.5−	1/20−	1/30+	1/50+		1/20?	1/50−	1/150−
3 C:isopropyl	1/10+	1/20+				1/10+	1/20+	
2 C:ethyl	1/20+					1/10+	1/20+	
2 C:xylene	1/20+					1/10+	1/20+	
1 C:toluene	1/20+	1/30+	1/50+			1/20+		
0 C:benzene	1/10+	1/20+				1/17.5+	1/20+	
(b) <i>Alkyl sulfates</i>								
12 C:dodecyl	1/2000−	1/5000−	1/10000±			1/666	contracture	1/1000 contracture
10 C:decyl	1/1000−	1/1500±				1/500−	1/1000−	
8 C:octyl	1/40−	1/120−	1/200±	1/400+		1/50−	1/150−	1/200−
7 C:diisopropylcarbinol	1/20+→	1/10+→				1/10+	1/12.5+	
6 C:2-ethylbutyl	1/10+	1/20+				1/10+	1/15+	1/20±
4 C:isobutyl	1/12.5+	1/40+				1/10+	1/20±	
(c) <i>Dialkyl sulfosuccinates</i>								
12 C:dihexyl MA	1/300−	1/500−	1/1000−			1/300?	1/500−	
10 C:diamyl AY	1/300−	1/500−	1/1000±			1/500−		
8 C:dibutyl IB	1/100−	1/300+→	1/500+			1/50−	1/100−	1/300+

apt to loosen the surface substrate of activity to the degree where it paralyzes the machinery, and after local application to produce an injury potential. But

⁴ However, this interpretation is not quite convincing because several additional observations obscure this picture. Although the rise of contraction height as well as the positive potential change is regularly present when adding a short chained detergent (see Table a-c), the rise of contraction with certain substances is not the only

that positivity is indicative of persistent excitability and as such a counterpart of the abolition of excitability by negativity. This is indeed the factual behavior. Following the aforementioned *Stromchentheorie* of Hermann (p. 114), the wave-like propagation of excitation is based upon the repetitive formation of the small electric circuits (see Fig. 1); in other words, it is based upon the repetitive local depolarization and repolarization of the membrane. As mentioned before, it is easy to understand that in the presence of the detergents with long alkyl chains the chances of repolarization are small or nil and different principally from those established by the addition of short chained detergents. Under normal conditions the surface potential of a resting nerve or muscle fiber is positive outside and is believed to be so because of the sieve-like character of the membrane with $K_i > K_o$ and the substance of the membrane bearing naturally a negative charge. Then, according to Michaelis a thermodynamical potential exists all around the fiber. But, upon the local addition of detergent and due to the adsorption of its anions, the negativity of the membrane substance rises even more; the thermodynamical potential locally increases, a reverted resting potential appears. Consequently, detergent anions are forced locally into the wall, the array of membrane micelles is loosened, and the entire structure more hydrated, but not loosened enough to cause falling apart. It follows that the excitation wave still can persist, and that local circuit currents still may arise, which are strong enough to reconsolidate the previously softened part of the surface. In this way there may be visualized the fact that an excitation wave can pass between two electrodes, one located inside the axoplasm of a giant nerve fiber, the other outside and opposite to the inner electrode, causing the ohmic resistance of the membrane between the two electrodes to drop down and immediately to rise again to its former value (Cole and Curtis (12)).

SUMMARY

In a previous paper it has been shown that the nonpolar-polar anionic detergents can be divided into two main groups. One chemically characterized by a relatively long chain of non-polar alkyl groups, which in solution are in contact with one end of a muscle and, locally adhering to it, produce a permanent negative injury potential. This is generally accompanied by a loss of excitability. The second group, distinguished by a relatively short chain of non-polar alkyl groups acts reversibly, ordinarily preserves the excitability and, in contrast to the first group, produces locally a reverse positive potential. For reasons mentioned before, this appears likely to correspond to an increased activity.

These concepts have been tested in this second paper. The measurements of the resting potentials of muscles have been supplemented by measurements on frog sciatics with the result that there are brought about, again by the

detergents with long alkyl chains, regular irreversible negative resting potentials and with the short chain compounds reversible positive potentials are aroused. Furthermore, in addition there appeared the hardly expected result that muscle stimulated in the presence of short chain detergents responded with an even higher contraction. We have endeavored to explain this on the basis of general considerations concerning the physical chemistry of the excitatory process.

More direct evidence of this rise of excitability under the influence of the short chain non-polar-polar detergents will be presented in the next papers on studies concerning chronaximetric measurements on nerve, referring particularly to the semidetergents, and concerning the effects of detergents in general upon the heart beat of a clam.

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OBSERVATIONS ON THE MOTILITY OF RABBIT SPERMATOZOA IN DILUTE SUSPENSION

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INTRODUCTION

Chang (1942) observed that the motility and respiration of rabbit spermatozoa are very low in dilute suspensions. This finding is reflected in the greater fertilising capacity of a constant number of spermatozoa when inseminated in small, as opposed to large volumes of diluent (Chang, 1946). Thus, an insemination with a total of 30,000 to 44,000 spermatozoa suspended in 1.0 ml. of saline was followed by cleavage in 0 per cent to 6 per cent of ova, whereas the same number of cells suspended in 0.1 ml. produced cleavage in 17 per cent to 42 per cent of ova, the difference being statistically significant. This "dilution phenomenon" is reminiscent of Milovanov's (1934 *a, b*) test of the resistance of an ejaculate, in which semen is rapidly diluted with a 1 per cent solution of sodium chloride until motility ceases; the index of resistance, R , is the volume of diluent required to produce immotility divided by the original volume of semen used in the test. Milovanov attributes this immobilising action to the toxicity of sodium chloride, but this point will be discussed below.

Our own observations, made in the course of an investigation of the release of the enzyme hyaluronidase from spermatozoa (Swyer, 1947) confirmed the rapid immobilisation of rabbit spermatozoa suspended in Baker's (1931) solution at a density of about half a million cells per ml., and the present communication deals with the results of various experiments initially designed to discover methods of maintaining motility under such conditions, and later extended in an attempt to explain the action of substances found to be effective. The need for dealing with suspensions of this order arose in the first place in attempts to discover whether spermatozoa which had been depleted of their hyaluronidase could reabsorb it on being placed in a medium rich in the enzyme. The question will receive some consideration in this paper.

Material and Methods

Active ejaculates with high initial motility in control suspensions in Baker's solution were used in the tests. These control suspensions were at 20 million cells per ml., a sufficient concentration to maintain as good or nearly as good a motility as in more dense suspensions. In testing the effect of dilution, various less concentrated suspensions were made by direct dilution from the control tubes. All tubes were kept covered and at room temperature, but were not under anaerobic conditions.

Motility was scored according to the method of Emmens (1947), in which fully active suspensions are given a rating of 4, suspensions exhibiting translational motility rate between 2 and 4 according to the degree of activity exhibited, while those exhibiting a preponderance of non-progressive movements rate from 0 to 2. It is frequently possible to interpolate to quarter-grades, and a motility score may therefore be given as a decimal fraction in the tables. The standard times of observation are $\frac{1}{2}$, $1\frac{1}{2}$, $2\frac{1}{2}$, 4, and 6 hours after the start of a test, sometimes with additional observations at 24 and 48 hours. An over-all index of motility is obtained for any one suspension by adding the individual scores for the whole period of the test, usually the five scores from $\frac{1}{2}$ to 6 hours. The use of this index as a variate is discussed in a previous paper (Emmens, 1948), where it is shown to be suitable for the analysis of variance. When, as in the 0.4 million per ml. dilutions of the present tests, motility sometimes falls rapidly to zero, the variance of the index is probably not completely independent of the level of response. This might result in unreliable significance tests for results on the borderline of significance, but is of little consequence when, as in the following tables, probabilities of less than 0.001 are most frequently in question. A standard test consists of observations on two ejaculates, with one tube from each ejaculate at every dilution and treatment tested. Such a test is normally found to be of ample sensitivity in determining the influence of treatments on motility.

RESULTS

(a) *The Effect of Progressive Dilution.*—Fig. 1 shows the mean motility index at various times in four semen specimens diluted to 20, 2.84, 0.41, and 0.06 million per ml. At the highest dilution, all motility was lost within 4 hours—in fact, in three of the four specimens it was lost within $2\frac{1}{2}$ hours. The analysis of variance for these data is given in Table I, in which the motility index for the 6 hour period is used as the variate. From Table I it is seen that the ejaculates behaved similarly in exhibiting a highly significant fall in motility with progressive dilution as compared with the less dilute suspensions. The most dilute suspension was not easy to work with, as so few spermatozoa were present per microscopic field. It was therefore decided to employ a dilution of 0.4 million per ml. as the standard “dilute” suspension.

(b) *The Effect of Chloride-Free Diluents.*—Milovanov (1934 a, b) finds that chlorides are injurious to spermatozoa and recommends the following chloride-free diluents for rabbit ejaculates:

SGR-2, which contains 39.0 gm. of anhydrous glucose, 3.5 gm. of anhydrous sodium sulfate, and 2.0 gm. of salt-free peptone per litre.

TGR-2, which contains 7.0 gm. of sodium potassium tartrate in place of the sodium sulfate in SGR-2.

A standard test with two ejaculates was therefore made of the effect of dilution with these diluents, and with variants in which the peptone was omitted, in comparison with Baker's solution (Baker's solution contains 2.0 gm. per litre of sodium chloride). The results are shown in Table II, in which the peptone-free variants have the suffix *a*.

It is clear from Table II that any effect of the chloride-free diluents on motility is deleterious rather than helpful at a concentration of 20 million per ml.,

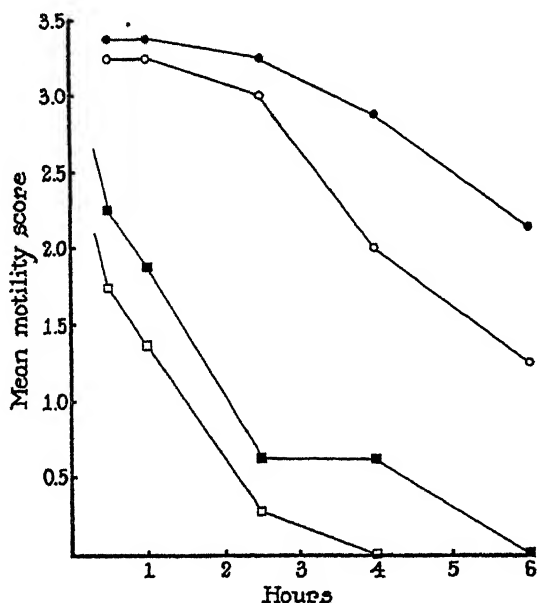


FIG. 1. Mean motility scores for suspensions of rabbit spermatozoa at different concentrations in Baker's solution.

● — ● 20 million per ml. ○ — ○ 2.9 million per ml.
 ■ — ■ 0.41 million per ml. □ — □ 0.06 million per ml.

TABLE I

Analysis of Variance for the Data Shown in Fig. 1

Source of variation	Degrees of freedom	Mean square	Variance ratio	P*
Differences between ejaculates.....	3	13.6	—	—
“ “ dilutions.....	3	117.5	26.3	<0.001
Interaction (error).....	9	4.48		

* The probability of the observed effects being due to chance. In this and the following tables, significant results are in bold-face type.

definitely so at 24 hours. On the other hand, some motility persisted until 6 hours in SGR-2, at a dilution of 0.4 million per ml., while the controls had become immotile rather earlier. The analysis of variance in Table III shows, however, that no significance is to be attached to the small variation which is

seen from diluent to diluent in the higher dilutions, as the mean square for differences between diluents is less than that for error.

It is concluded, therefore, that the immobilising effect of high dilution in Baker's solution is not to be attributed to its content of sodium chloride and that this diluent is more suitable than SGR-2 and TGR-2 for the study of the

TABLE II

The Effect of Dilution of Rabbit Ejaculates in Chloride-Free Media. Each Figure Is the Mean of Two Replicates (for Analysis see Table III)

Diluent	Sperm/ml.	Mean motility score at hrs.					
		$\frac{1}{2}$	$1\frac{1}{2}$	$2\frac{1}{2}$	4	6	24
	<i>millions</i>						
Baker (controls)	20	3.8	4.0	3.8	3.8	4.0	2.3
	0.4	1.5	1.0	0.3	0.0	0.0	0.0
SGR-2	20	3.3	4.0	3.8	3.5	3.1	0.1
	0.4	0.8	0.8	0.8	0.5	0.3	0.0
SGR-2a	20	3.4	4.0	3.8	3.5	3.1	0.3
	0.4	0.4	0.0	0.0	0.0	0.0	0.0
TGR-2	20	3.5	3.9	3.5	3.5	3.1	0.1
	0.4	1.0	0.8	0.5	0.5	0.0	0.0
TGR-2a	20	3.8	3.9	3.8	3.6	2.3	0.1
	0.4	1.5	1.5	1.3	0.5	0.0	0.0

TABLE III

Analysis of Variance for the 0.4 Million/Ml. Dilutions of Table II. Unit Observation Is the Added Motility Indices for the Complete Test

Source of variation	Degrees of freedom	Mean square	Variance ratio
Ejaculates	1	0.76	—
Diluents	4	4.84	0.68
Interaction (error)	4	7.16	

phenomenon, since activity persists better in Baker's solution at 20 million per ml. The poor quality of the chloride-free diluents is in line with their low initial pH (6.8 to 7.4 when mixed with semen) and instability of pH, which fell in 24 hours to between 5.2 and 6.4 in different tubes at 20 million per ml. In view of these findings the immobilising activity of 1 per cent sodium chloride in Milovanov's test would seem to be due in part, if not in entirety, to the effect of dilution itself, at any rate with rabbit spermatozoa. To test this pos-

sibility, determinations of R were made by Milovanov's method with eleven different ejaculates, using physiological saline, SGR-2, and Baker's solution in random order with each ejaculate. The results are shown in Table IV. When an ejaculate was tested twice with the same diluent, the first test, labelled a , preceded the second test by between 1 and 3 hours. It is seen that aging of the specimen tends to lower the value of R .

The results in Table IV clearly demonstrate that the immobilising effect in Milovanov's test is not due to sodium chloride, as SGR-2, the chloride-free diluent, is more often worse than physiological saline for preserving motility

TABLE IV
Determinations of Milovanov's Factor "R" for Rabbit Ejaculates

Sample No.	Saline	Diluent: SGR-2	Baker
1	900	900	4,700
2	<100	<100	300
3	2,200	5,500	>10,000
4	4,000	<100	4,000
5	>20,000	9,200	>20,000
6	600	<100	400
7a	16,000	>20,000	>20,000
7b	6,000	8,000	10,000
8a	3,200	3,200	8,000
8b	2,000	2,000	5,000
9a	>20,000	>20,000	>20,000
9b	17,000	1,600	>20,000
10a	>20,000	>20,000	>20,000
10b	>20,000	5,000	>20,000
11	>20,000	>20,000	>20,000

a = 1st test, b = 2nd test, 1 to 3 hours later.

than the reverse. The use of Baker's solution usually permits of greater dilution before immotility occurs than do either of the other diluents.

(c) *The Effect of Catalase*.—There is strong evidence that spermatozoa, at least those of man, ram, and bull, do not contain catalase in significant amounts (MacLeod, 1943; Tosic and Walton, 1945; Tosic, 1946; Blom and Christensen, 1947; Mann, 1948) and it was thought that the production of toxic amounts of hydrogen peroxide in the presence of a relative excess of dissolved oxygen might explain the rapid fall in motility in dilute suspension. It has already been shown that semianaerobic conditions do not decrease the rate of fall in motility of concentrated suspensions (Emmens, 1948) and that production of hydrogen peroxide is not important at these concentrations. The ineffectiveness of added catalase (a highly purified preparation kindly supplied by Dr. T. Mann) is shown in Table V, for which an analysis of variance is clearly superfluous.

In further tables, only the 4 and 6 hour indices will be given, a high index necessarily indicating that the spermatozoa survived at good motility during the test period. The maximum possible value of a 4 hour index is 16, and of a 6 hour index is 20.

(d) *The Effect of Oil Emulsions and of Supernatants from Other Suspensions.*—Walton (personal communication) has observed that an emulsion of liquid paraffin in ordinary diluents helps to preserve motility. It was therefore decided to test the effect of emulsions of from 0.25 to 0.5 per cent liquid paraffin, emulsified with the aid of a little gum arabic, on the dilution phenomenon. In addition, it was thought that a possible effect of dilution might be the excessive loss of some essential material, such as an intracellular enzyme, and that this loss might be prevented by using spermatozoa-free supernatants from relatively dense suspensions in place of the usual Baker's solution. This type of supernatant (SA in Table VI) was prepared by centrifuging a suspension of two

TABLE V

The Absence of an Effect of Catalase on the Fall in Motility of Rabbit Spermatozoa in Dilute Suspension

Sperm/ml.	Catalase	No. of ejaculates	Mean motility score, at hrs.				4 hr. index (totals)
			$\frac{1}{2}$	$1\frac{1}{2}$	$2\frac{1}{2}$	4	
<i>millions</i>	<i>per cent</i>						
20	0.0	3	3.7	3.5	3.3	3.2	13.7
0.4	0.0	3	1.5	0.3	0.0	0.0	1.8
0.4	0.01	3	1.0	0.5	0.5	0.0	2.0
0.4	0.1	1	2.0	0.0	0.0	0.0	2.0

ejaculates in 40 ml. of Baker's solution after leaving it to stand for 40 minutes (test 1, Table VI) or overnight (all other tests). Since the leaching of hyaluronidase from spermatozoa is most effectively realised by freezing (Swyer, 1947) a further supernatant was made from a similar suspension frozen overnight (SB). Test 1, Table VI, showed the effectiveness of an oil emulsion and of supernatant SA, hence other supernatants were prepared (1) from a suspension of two ejaculates in 40 ml. of an oil emulsion prepared as above (SC), in case this *prevented* the escape of the hypothetical factor from the cells, (2) by boiling an SA supernatant (SD), in case the activity was destroyed by heat, and (3) from a suspension of the aspermic ejaculate from a vasectomised buck (SV), in case the activity was not associated with the presence of spermatozoa at all. In test 4, Table VI, this "supernatant" was not in fact centrifuged, and consisted of a suspension of the ejaculate in 20 ml. of Baker's solution. The motility indices (4 or 6 hour) for duplicate sets of tubes from different ejaculates are shown in Table VI. The analyses of variance for the data in Table VI are given in Tables VII and VIII. The mean squares for error differ in the

two dilutions, that for 0.4 million per ml. is the greater, but they do not differ significantly from test to test within each dilution, and have hence been combined to give an over-all estimate based on more degrees of freedom than are available in each separate test.

The standard error of an observation at 0.4 million per ml. is 1.75, which is also the standard error of the difference between the means of two pairs of duplicates. With an estimate of error based on 14 degrees of freedom, the

TABLE VI

4 or 6 Hour Motility Indices from Duplicate Tests (Two Ejaculates) of the Effect of an Oil Emulsion and Various Supernatants on the Motility of Rabbit Spermatozoa (for Explanation See Text)

Diluent	Concentration of sperm millions/ml.	Motility indices for duplicates in test							
		1 (4 hr.)		2 (4 hr.)		3 (6 hr.)		4 (6 hr.)	
Baker	20	15.5	14.5	13.0	14.0	17.5	20.0	19.0	20.0
"	0.4	0.0	1.5	0.0	1.5	0.0	2.0	0.0	3.5
SA	20	15.5	14.8	13.0	14.8	17.0	20.0	20.0	20.0
"	0.4	9.5	6.0	10.0	11.5	15.5	19.8	10.0	17.0
SB	20	—	—	14.0	14.8	—	—	—	—
"	0.4	—	—	12.0	12.0	—	—	—	—
SC	20	—	—	14.0	14.8	—	—	—	—
"	0.4	—	—	12.0	12.0	—	—	—	—
SD	20	—	—	—	—	18.0	20.0	—	—
"	0.4	—	—	—	—	16.0	17.0	—	—
SV	20	—	—	—	—	17.5	20.0	20.0	20.0
"	0.4	—	—	—	—	14.5	13.5	7.5	11.0
" dil. 1/5*	0.4	—	—	—	—	—	—	6.0	9.0
" " 1/25*	0.4	—	—	—	—	—	—	4.5	4.0
Oil emulsion	20	15.5	12.0	—	—	18.0	20.0	19.8	20.0
" "	0.4	14.0	13.0	—	—	18.0	18.0	16.0	13.5

* Dilutions of 1 in 5 and 1 in 25 of the supernatant in Baker's solution.

mean difference between two such pairs must be at least 3.8 ($P = 0.05$) or 5.2 ($P = 0.01$) for significance, where P is the probability that the difference has arisen by chance alone. Thus, all three treatments in test 1 differ significantly, while the significance of the action of all supernatants and of oil emulsion in preserving motility at high dilution is in no practical doubt whatever. It may be noted that the significantly less favourable action of SA in test 1, as compared with oil, was not confirmed either for SA itself or the variants, SB, SC, and SD in later tests, and that this particular result is probably attributable to the short time during which the suspension from which the supernatant was prepared was allowed to stand.

TABLE VII

Analyses of Variance for the 20 Million per Ml. Dilutions of Table VI

Test No.	Source of variation	Degrees of freedom	Mean square	Variance ratio	P*
1	Ejaculates	1	4.60	—	—
	Diluents	2	1.16	3.9	= 0.05
2	Ejaculates	1	5.16	—	—
	Diluents	3	0.70	2.3	> 0.05
3	Ejaculates	1	14.62	—	—
	Diluents	4	0.09	0.3	> 0.05
4	Ejaculates	1	0.28	—	—
	Diluents	3	0.11	0.4	> 0.05
1-4	Interaction (error)	12	0.300		

* The probability that the observed effect is due to chance.

TABLE VIII

Analyses of Variance for the 0.4 Million per Ml. Dilutions of Table VI

Test No.	Source of variation	Degrees of freedom	Mean square	Variance ratio	P*
1	Ejaculates	1	1.50	—	—
	Diluents	2	81.54	26.6	< 0.001
2	Ejaculates	1	1.12	—	—
	Diluents	3	59.39	19.4	< 0.001
3	Ejaculates	1	3.60	—	—
	Diluents	4	100.58	32.8	< 0.001
4	Ejaculates	1	16.32	—	—
	Diluents	5	258.50	84.5	< 0.001
1-4	Interaction (error)	14	3.06		

* The probability that the observed effect is due to chance.

The conclusions to be drawn from these tests are self-apparent. At a dilution of 20 million per ml., no difference is seen as a result of any treatment, *i.e.* the various diluents were neither toxic nor, where there was scope for it, beneficial. At a dilution of 0.4 million per ml. no difference is seen between the various supernatants from a suspension of spermatozoa and an oil emulsion, except the relative inefficiency of SA in test 1 already noted. SV, however,

was not as effective as the others, although it had some action. The lower efficiency of SA and SV would seem to indicate that seminal plasma alone is not sufficient for the maintenance of full motility over the 4 or 6 hours of the test, and that a further contribution comes from the spermatozoa themselves. The substance (or substances) in a supernatant responsible for the maintenance is, however, heat-stable.

In tests 2, 3, and 4 (Table VI), the motility of 0.4 million per ml. suspensions in supernatants SA-SD approached, but did not equal that of the corresponding 20 million per ml. suspensions. Without labouring the analysis, it may be noted that in all of twelve cases the motility index was less in the 0.4 million per ml. tubes. This would occur by chance only once in 4096 times if there were no real difference. A further observation taken at 24 hours in each test amplifies this conclusion, the mean motility rating of all twelve more concentrated suspensions then being 1.8, and of all twelve less concentrated suspensions 0.95. Thus, it is further concluded that, despite the considerable effect of supernatants in maintaining motility, this maintenance is not complete in comparison with controls at a higher concentration. The same, although less well substantiated conclusion seems to hold for the oil emulsion as well.

(e) *The Effect of Various Other Diluents.*—The fact that liquid paraffin emulsified with the aid of gum arabic and the various supernatants were all effective in prolonging motility in dilute suspensions suggested that the action might not be very specific. A number of substances which happened to be at hand or easily obtainable were therefore tested; the choice of substances was in part guided by previous work and in part by a desire to test widely different materials. They were dissolved in Baker's solution, and the results of three successive tests are given in Table IX. The only suspensions at 20 million per ml. included in these tests were the controls in Baker's solution. Summary analyses of variance for the tests are given in Table X, a common error being used as before, since the individual estimates of error were again homogeneous when tested statistically. The standard error of the difference between the means of two pairs of duplicates is 1.82. With an estimate of error based on 24 degrees of freedom the mean difference between two such pairs must be at least 3.8 ($P = 0.05$) or 5.1 ($P = 0.01$) for significance.

It is seen that the last twelve substances in Table IX had a significant effect on motility ($P < 0.01$ in all but the case of dextrin), whereas agar, gelatin, and sodium silicate were without effect. In test 3, all solutions were boiled for 15 minutes before the test (the starch, agar, and gelatin solutions in test 1 were also boiled), demonstrating once more the stability of the responsible agents to heat. The proteins were crystalline, Nos. 1, 2, and 3 being globo-glycoid preparations kindly provided by Professor C. Rimington, No. 4 being a preparation of crystalline bovine serum albumin prepared by Armour Laboratories, Chicago, Illinois, and they did not precipitate on heating in the concentration used. It

TABLE IX

The Influence of Various Purified and Impure Substances on the Dilution Phenomenon (6 Hour Tests in Duplicate)

Diluent	Concentration of sperm.	Motility indices for duplicates in test					
		1		2 (unboiled)		3 (boiled)	
<i>per cent are W/V*</i>	<i>millions/ml.</i>						
Baker	20	15.0	13.5	20.0	14.5	20.0	11.3
"	0.4	0.0	0.5	1.5	0.0	0.0	1.3
0.1 per cent agar	0.4	0.0	0.5	—	—	—	—
0.25 per cent gelatin	0.4	0.0	2.5	—	—	—	—
0.25 per cent Na silicate	0.4	0.3	1.5	—	—	—	—
0.25 per cent gum arabic	0.4	7.5	13.3	—	—	—	—
0.25 per cent starch	0.4	10.8	12.0	—	—	16.0	8.5
0.25 per cent dextrin	0.4	—	—	—	—	9.5	4.5
0.25 per cent inulin	0.4	—	—	—	—	11.5	6.5
0.25 per cent glycogen	0.4	—	—	16.0	10.0	16.3	10.5
1.0 per cent egg white	0.4	11.5	11.8	—	—	—	—
1.0 per cent egg yolk	0.4	10.3	11.3	—	—	—	—
0.25 per cent CHO-free protein (1)	0.4	—	—	13.5	13.5	16.5	12.0
0.25 " " " " (2)	0.4	—	—	12.5	11.5	12.8	8.5
0.25 " " CHO-7 per cent protein (3)	0.4	—	—	12.5	11.0	15.5	12.8
0.25 per cent bovine serum albumin (4)	0.4	—	—	10.0	11.3	11.5	11.0
0.25 per cent oil (no gum arabic)	0.4	—	—	7.0	5.5	—	—

* W = weight of solute; V = volume of final solution.

TABLE X

Analyses of Variance for the Data of Table IX

Test No.	Source of variation	Degrees of freedom	Mean square	Variance ratio	<i>p</i> *
1	Ejaculates	1	7.4	—	—
	Diluents	8	70.0	21.2	<0.001
2	Ejaculates	1	15.5	—	—
	Diluents	7	50.7	15.3	<0.001
3	Ejaculates	1	93.5	—	—
	Diluents	9	39.5	11.9	<0.001
1-3	Interaction (error)	24	3.31		

* The probability that the observed effect is due to chance.

is once more apparent that most, if not all of the substances tested are not as effective as is a higher concentration of spermatozoa in Baker's solution in

maintaining motility. The effectiveness of proteins (2) and (4), oil emulsion not made with gum arabic, dextrin, and inulin, is, in each case, almost certainly less than optimal. Thus, the action of the previous oil emulsions can be largely attributed to their content of gum arabic. Nevertheless, the widespread capacity of this heterogeneous collection of substances to mitigate the lethal influence of dilution makes it reasonably certain that a physical rather than a chemical action is concerned.

The effectiveness of none of the substances in Table IX continued at a high level for 24 hours. By this time the motility of suspensions at 20 million per ml. ranged from $1\frac{1}{2}$ to 3, while the more dilute suspensions were in all cases immotile or nearly immotile. This is in agreement with the previous findings with oil emulsions and supernatants.

TABLE XI

The Influence of Purer Diluents on the Dilution Phenomenon (6 Hour Tests with Two Ejaculates)

Diluent (see text)	Concentration of sperm <i>millions/ml.</i>	Motility indices for duplicates			
		Untreated		"Norit"-treated	
Baker (1) and (2)	20	19.5	18.0	20.0	20.0
" " " "	0.4	0.0	0.0	3.5	2.0
Baker (3) and (4)	20	20.0	17.5	20.0	20.0
" " " "	0.4	6.5	2.5	1.0	1.8
Cu Baker (5)	20	20.0	18.5	—	—
" " " "	0.4	0.0	0.0	—	—

(f) *The Effect of Heavy Metals.*—In view of the decision that the action of the various effective agents is physical, the possibility was next explored that they might adsorb a toxic agent present in Baker's solution as normally prepared with analar reagents and distilled water made with a copper still. Such agents as traces of copper, lead, or arsenic in the reagents might prove more lethal to dilute suspensions, although chemical tests for the presence of these metals in the diluents were negative. A test was therefore made with the following diluents:

(1) Baker's solution as normally prepared; (2) The same solution treated with Norit brand-activated charcoal overnight and filtered; (3) Baker's solution made with water doubly distilled over glass; (4) The same solution treated as (2); (5) Baker's solution (1) with 10 parts per million of copper added as copper sulfate.

The results of a standard test are shown in Table XI, and the analysis of variance in Table XII.

The apparent effect of treatment with activated charcoal is seen to be contradictory in the two preparations of Baker's solution, but on statistical analysis it is further seen that no significance can be attached to the differences between diluents at either concentration. It is not certain that the charcoal used was itself metal-free, but since the presence of more than a trace of copper had no influence on motility at 20 million per ml. it was thought more profitable to investigate the effect of relatively high concentrations of copper, lead, and arsenic, than to attempt to purify the diluents more thoroughly. The presence of mercury, known to be highly toxic to spermatozoa, was not in question.

The next test, therefore, consisted in the investigation of motility in Baker's solution containing up to 160 parts per million of copper or lead, or up to 48 parts per million of arsenic. These concentrations are far beyond those in the reagents used in preparing the diluents. The diluents were used for preparing

TABLE XII
Analyses of Variance for the Data in Table XI

Concentration of sperm	Source of variation	Degrees of freedom	Mean square	Variance ratio	P*
<i>millions/ml.</i>					
20	Ejaculates	1	3.02	—	—
	Diluents	4	0.79	1.3	>0.05
	Interaction (error)	4	0.59		
0.4	Ejaculates	1	2.26	—	—
	Diluents	4	7.41	4.1	>0.05
	Interaction (error)	4	1.79		

* The probability that the observed effect is due to chance.

suspensions at 20 million per ml., copper being added as the sulfate, lead as the nitrate, and arsenic as potassium arsenite. Motility indices for a 6 hour test and the motility score at 24 hours are shown in Table XIII, which lists the results of a standard test.

The results are unequivocal in showing no toxicity during the first 6 hours at any concentration of metal, but a toxic influence of copper by 24 hours. This surprising eventuality makes it unlikely that the rapid immobilisation of spermatozoa by dilution can be due to the toxic action even of copper, which is not present in Baker's solution as normally prepared to the extent of 1 part per million.

(g) *The Effect of Repeated Washing.*—A further method of investigating the possibility that spermatozoa in dilute suspension lose vital material excessively is to wash them repeatedly in fresh diluent. If the maintenance of activity depends on the continued presence of seminal plasma, a few washings, which would remove practically all of this, should result in rapid immobilisation. If

it depends on the leaching of internal material from the cells many washings might be required, as the loss of this material would depend both on its concentration in the surrounding medium and on the duration of exposure to the medium. Consequently, two tests were made; in the first only two washings were given; in the second six washings were given.

The first test involved two ejaculates, each of which was suspended at 20 million per ml. in Baker's solution and centrifuged for 30 minutes. The supernatants were removed and each ejaculate resuspended in the original volume of fresh diluent and divided into two equal portions. These were again centrifuged and the supernatants removed. Then one half of each ejaculate was re-

TABLE XIII

The Influence of Heavy Metals on the Motility of Rabbit Spermatozoa at 20 Millions per Ml. in Baker's Solution (Duplicate Tubes with Two Ejaculates)

Metal	Concentration (parts per million)	6 hr. motility indices		24 hr. motility scores	
Nil	—	13.8	14.3	2.5	3.5
Cu	10	13.0	11.5	0.5	0.0
	40	14.0	15.8	1.0	0.0
	160	15.5	13.8	0.3	0.0
Pb	10	15.0	18.3	2.8	3.5
	40	15.5	16.0	2.8	3.0
	160	14.8	12.8	2.5	2.0
As	0.75	14.3	17.8	2.5	3.5
	3	14.5	18.0	2.5	3.0
	12	15.5	19.5	2.0	2.5
	48	14.5	18.0	1.5	2.5

suspended in fresh diluent, while the other half was suspended in the original first supernatant. Thus, twice washed spermatozoa were placed either in a solution of seminal plasma equivalent to that produced in making the original dilution to 20 million per ml. (1 in 32.5 and 1 in 17.5 respectively), or in fresh diluent. Allowing for the residual plasma left behind when removing the supernatants, the concentration of plasma in the two latter suspensions was approximately 1 in 960 and 1 in 525 respectively. There was no influence of washing on motility. The motility scores of the first ejaculate were 4 in both suspensions up to 6 hours from the last resuspension, and $3\frac{1}{2}$ at 24 hours. Those of the second ejaculate were $3\frac{1}{2}$ or 4 in both tubes up to 6 hours, and 2 and $2\frac{1}{2}$ at 24 hours, the spermatozoa suspended in fresh diluent exhibiting the higher motility at 24 hours. Thus, spermatozoa in relatively plasma-free suspensions

do not exhibit the "dilution phenomenon" when kept at a concentration of 20 million per ml., although the concentration of seminal plasma is approximately equal to that occurring in a suspension at 0.4 million per ml. prepared by direct dilution.

The second test (Table XIV) commenced with the same procedure as before, four ejaculates being used, with the modification that each suspension was divided into two from the start, and in the control tubes, labelled *a* in Table XIV, the spermatozoa were resuspended by agitation in the same unreplaced diluent. This time, six washings were made in the course of $2\frac{1}{2}$ hours, at the end of which the washed spermatozoa were split into two portions, one, labelled *b* was suspended in fresh diluent while the other, labelled *c*, was suspended, in

TABLE XIV
The Effects of Repeated Washing on the Motility of Rabbit Spermatozoa

Time <i>hrs.</i>	Washing No.	Motility indices for ejaculates									
		1a	1b	2a	2b	3a	3b	3c	4a	4b	4c
$\frac{1}{2}$	1	$3\frac{1}{2}$	$3\frac{1}{2}$	$3\frac{1}{2}$	$3\frac{1}{2}$	4	4	—	4	4	—
1	2	$2\frac{1}{2}$	2	3	$2\frac{1}{2}$	4	$3\frac{1}{2}$	—	$3\frac{1}{2}$	$3\frac{1}{2}$	—
$1\frac{1}{2}$	3	2	$1\frac{1}{2}$	$2\frac{1}{2}$	$2\frac{1}{2}$	$3\frac{1}{2}$	$2\frac{1}{2}$	—	$3\frac{1}{2}$	$2\frac{1}{2}$	—
$1\frac{3}{4}$	4	$1\frac{1}{2}$	1	2	2	3	2	—	$3\frac{1}{2}$	$2\frac{1}{2}$	—
2	5	2	1	2	1	3	2	—	$3\frac{1}{2}$	2	—
$2\frac{1}{2}$	6	2	$1\frac{1}{2}$	2	$\frac{1}{2}$	3	2	$2\frac{1}{2}$	$3\frac{1}{2}$	$1\frac{1}{2}$	3
$3\frac{1}{2}$	—	$2\frac{1}{2}$	2	$2\frac{1}{2}$	$1\frac{1}{2}$	$3\frac{1}{2}$	$2\frac{1}{2}$	$3\frac{1}{2}$	$3\frac{1}{2}$	1	$3\frac{1}{2}$
$4\frac{1}{2}$	—	$2\frac{1}{2}$	$1\frac{1}{2}$	$2\frac{1}{2}$	1	$3\frac{1}{2}$	$1\frac{1}{2}$	$3\frac{1}{2}$	4	1	4
$5\frac{1}{2}$	—	3	$1\frac{1}{2}$	$2\frac{1}{2}$	$\frac{1}{2}$	4	$1\frac{1}{2}$	4	4	$1\frac{1}{2}$	4
$6\frac{1}{2}$	—	3	1	$2\frac{1}{2}$	$\frac{1}{2}$	4	$\frac{1}{2}$	4	4	1	4

the case of ejaculates 3 and 4, in a supernatant from a 1 in 10 dilution of another ejaculate which had stood for about half an hour before centrifuging. From Table XIV it is clear that, while repeated centrifuging depressed motility and some recovery took place on resting in all suspensions, the motility of 6 times washed spermatozoa was almost abolished unless they were then resuspended in a supernatant rich in seminal plasma, when full motility was regained. Thus, dilution of the plasma to an extent which, in a correspondingly dilute suspension of spermatozoa, results in rapid immobilisation, does not so immobilise a more concentrated suspension. Sufficient washing of the more concentrated suspension results in a loss of motility which is fully regained on the addition of seminal plasma. This argues both for loss of intracellular material as a cause of the dilution phenomenon and for its sufficient replacement on suspension in a medium containing it—if indeed it is necessary for such material to be reabsorbed in order to exert its specific effect. That such reab-

sorption can take place is demonstrated for hyaluronidase by the experiment described below.

(h) *The Reabsorption of Hyaluronidase by Spermatozoa.*—Hyaluronidase-containing solutions consisted of (1) a supernatant from 10 ml. of Baker's solution containing 0.2 ml. of rabbit semen frozen overnight and centrifuged, and (2) 10 ml. of Baker's solution containing 10 units of bull semen filtrate (Swyer and Emmens, 1947). The rabbit semen to be tested was suspended in two 0.2 ml. aliquots in 100 ml. of Baker's solution containing 0.25 per cent (W/V) gum arabic to prevent the dilution phenomenon. After 1 hour the suspensions were centrifuged for half an hour and the supernatants were removed. The spermatozoa were then resuspended in 5 ml. of solutions (1) and (2) and again left for 1 hour before centrifuging for half an hour. The supernatants were removed and stored frozen until assayed by the viscosimetric method (Swyer and Emmens, 1947).

These final supernatants were assayed against solutions (1) and (2) respectively; the estimate of potency of solution (1) in terms of the corresponding final supernatant was 1.37, fiducial limits of error ($P = 0.05$) 1.15 to 1.61, demonstrating that the spermatozoa had reabsorbed a significant amount of homologous hyaluronidase. There was, however, no significant difference in potency between solution (2) and the corresponding final supernatant, indicating that rabbit spermatozoa cannot absorb heterologous (bull) hyaluronidase.

The approximate quantities of enzyme involved in the test of the reabsorption of homologous hyaluronidase were:

0.2 ml. of ejaculate contained a total of	10.0 units
The 100 ml. of Baker's solution in which it was suspended gained	5.0 units
Loss by spermatozoa	5.0 units
The 5 ml. of solution (1) in which they were resuspended con- tained	7.7 units
After suspension it contained	5.6 units
Gain by spermatozoa	2.1 units

These figures are approximate, because assay in terms of bull semen filtrate, the standard preparation, was in part invalidated by an unexplained significant difference in slope between the dose/response lines. This does not affect the conclusion that the spermatozoa absorbed homologous hyaluronidase, since this was demonstrated by direct comparison between rabbit semen supernatants in a fully valid assay.

DISCUSSION

Tests designed to discover means of maintaining the motility of spermatozoa in high dilution have revealed an abundance of effective or partially effective

agents. A certain amount of discussion has been interpolated in presenting results, where it seems more readily digestible and avoids repeated back reference. It is proposed only to emphasize here the outstanding points which have emerged.

Firstly, with reference to the constituents of semen, it would seem that the following fractions have differential activity:

1. The accessory secretions significantly prolong the motility of dilute suspensions, but under the conditions investigated their action is confined to a few hours.

2. Supernatants from suspensions of spermatozoa left overnight further prolong motility, yet their action is not equivalent to suspension at a higher concentration, and is largely dissipated within 24 hours. There is good evidence that these supernatants are more effective than seminal plasma alone because they contain material lost by spermatozoa on standing.

Secondly, it seems more than coincidental that the most potent of the various exogenous agents investigated behave as members of class (2) above, and do not maintain full motility for very long. Their action would seem to be physical, perhaps bound up in some way with the colloidal state, although the complete ineffectiveness of other colloids makes this assumption dubious. If one is conservative of hypotheses and supposes that a single ultimate agency is at work, the most likely suggestion would seem to be that these substances prevent the loss of vital material from the spermatozoa. The way in which they may do this is not apparent, and it is difficult to believe that a complex material, such as an enzyme system, could readily pass out of and into the cells. That hyaluronidase can undergo the postulated translations has already been shown, though there is no proof that it actually traverses the cell membrane in the process. Indeed, it would seem that the most likely explanation is that the effects in question are surface phenomena, so that adsorption rather than absorption would perhaps be the more accurate description. Recent electron microscope researches on the structure of spermatozoa (Bretschneider and van Iterson, 1947) have shown that the whole surface of the flagellum is covered by a fine helix of orientated molecules, and from analogy with muscle, it is likely that the motor mechanism is situated in these surface structures. Walton (1947) has drawn attention to the importance of surface phenomena in spermateliosis and spermatozoal physiology.

A somewhat speculative, but nevertheless not unreasonable alternative explanation for the action of the agents effective in mitigating the dilution phenomenon is the suggestion that they may all contain traces of a contaminant itself active in very minute concentrations. Such a situation would be analogous with that found with biotin, which could, indeed, be the hypothetical contaminant.

Milovanov (1934 *a, b*) states that it has been clearly demonstrated by Selivanova that the "lipoid capsule" of the spermatozoon is destroyed by the acces-

sory secretions, and that there is an inverse relationship between the amount of accessory secretion in the semen and the duration of life of the spermatozoa. On the other hand, he finds that gum, gelatin, egg albumin, meat or blood serum albumin, and mucin from boar semen protect spermatozoa in descending order of activity. The findings reported here do not substantiate Milovanov's statement about the accessory secretions, nor is gelatin found to be efficient in high dilutions of spermatozoa, but it must be stressed that Milovanov was discussing suspensions at high concentration. It remains possible, nevertheless, that the accessory secretions possess a stimulant action of short duration which is not conducive to the successful storage of spermatozoa over long periods.

Doubt has also been cast on the exact significance of Milovanov's test of resistance. Emmens (1948) has shown that sodium chloride is not harmful in isotonic diluents except at an alkaline pH, and the resistance measured in this test would seem to be resistance to the effect of dilution, not to that of sodium chloride.

There is ample evidence that marked species differences in the physiology of spermatozoa occur, and it is therefore possible that the behaviour of rabbit spermatozoa that has been described in this paper might not be reproduced by the spermatozoa of other species under comparable conditions. Nevertheless, the occurrence of the dilution effects with human spermatozoa (Kennedy, 1947) suggests that the phenomena in question are not confined to the rabbit. Kennedy repeated some of the experiments reported in this paper using human spermatozoa, and it is of interest to note that the range of spermatozoal densities over which the dilution phenomenon appears is of the same order for human as for rabbit spermatozoa.

SUMMARY

1. Rabbit spermatozoa suspended in Baker's solution rapidly lose motility at relatively high dilutions. At a concentration of 0.4 million per ml., the spermatozoa from most ejaculates are completely immotile within 2 or 3 hours. The same phenomenon occurs with chloride-free diluents and is therefore not due to the toxic action of chlorides.

2. This rapid immobilisation may be prevented by suspension in cell-free supernatants from other more concentrated suspensions of rabbit semen and by the accessory secretions from a vasectomised buck. The most effective supernatants are those prepared from suspensions of spermatozoa which have been left overnight before centrifuging. Immobilisation may also be prevented by many other agents, the most effective of which are gum arabic, starch, glycogen, and serum proteins (carbohydrate-containing or carbohydrate-free). Yet, in no case is the action of these substances as effective as more concentrated suspension in Baker's solution.

3. The immobilisation is not prevented by catalase, gelatin, agar, or sodium silicate. It is almost certainly not due to the toxic action of traces of heavy

metals and is not affected by the use of water doubly distilled over glass in the preparation of the diluent, or by treating the diluent with activated charcoal.

4. Washing with Baker's solution does not cause immobilisation of spermatozoa suspended at 20 million per ml. at a stage at which the concentration of seminal plasma has been reduced to that equivalent to dilution to 0.4 million per ml. Further washing (six repeated centrifugings of 0.2 ml. semen in 4 ml. of Baker's solution) immobilises them. This confirms the opinion that loss of intracellular, or perhaps rather paracellular, material is a responsible agent in the dilution phenomenon.

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THE STIMULATING EFFECT OF GLYCOLS AND THEIR POLYMERS ON THE TARSAL RECEPTORS OF BLOWFLIES*

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INTRODUCTION

Previous studies of the rejection thresholds of the blowfly *Phormia regina* Meigen for series of aliphatic alcohols have revealed a very high degree of correlation between the concentration at rejection and those chemical properties which are related thermodynamically to one another (3).

Because the technique of testing requires an aqueous solution, it had not been possible to extend the study of the relationship between chemical structure and stimulation beyond a chain length of eight carbon atoms. This difficulty has now been overcome in part by the use of water-soluble high polymers of the glycol series. The present study was undertaken, therefore, to examine the relation between stimulating effect and chemical properties with compounds having chain lengths up to C_{272} and to compare the effects of compounds containing ether linkages with the effects of straight saturated hydrocarbon chains.

The experimental technique was identical with that followed in earlier studies (3). For the purpose of statistical analysis the raw data were resampled in the following manner. A table with as many columns as there were concentrations tested was constructed for each compound. The specimens were now sampled, five at a time, in the order in which they had appeared for testing. For the first group of five, the number rejecting at the highest concentration was recorded in the table; for the next group, the number rejecting at the next lower concentration; and so on, in rotation, until all flies used in the test had been recorded. The percentages rejecting at each concentration were calculated from the totals in the columns, converted to probability units, and plotted against the logarithms of the respective concentrations. The most probable value of the log concentration rejected by 50 per cent of the flies was then determined according to the procedure described by Bliss (1) and has been used for comparing the responses of the population to the different compounds tested. The basis for this treatment of the results is considered in the discussion below.

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Ethylene, diethylene, triethylene, tetraethylene, dipropylene, ethylhexylene (2-ethyl hexanediol-1,3), and polyethylene glycols together with data on their properties were supplied through the courtesy of Carbide and Carbon Chemicals Corporation. Tripropylene glycol and three polypropylene glycols were received through the courtesy of the Dow Chemical Company. Propylene glycol was obtained from Eimer and Amend, trimethylene from Eastman, and decamethylene from Paragon Testing Laboratories. Octanediol-2,3 was supplied by the Orlando Laboratory of the Bureau of Entomology and Plant Quarantine, United States Department of Agriculture, through the cooperation of the Chemical-Biological Coordination Center of the National Research Council.

Ethylene, trimethylene, and tetraethylene glycols were further purified by redistillation at reduced pressure. With the first there was no significant difference between thresholds obtained with the crude and with the purified product. Trimethylene and tetraethylene glycols, however, gave significantly higher thresholds after distillation, and there is reason to suspect that the former still contained a certain amount of physiologically active impurities. These results suggested the desirability of testing the purity of each of the compounds used exclusive of the polymers; hence boiling point determinations were made.

Polyethylene and polypropylene glycols are mixtures of polymers, and the numerical designation of each mixture represents the average molecular weight. Molecular size distribution within such a mixture follows a Poisson distribution (6, 7). Attempts at fractionation for the purpose of isolating some of these species were not entirely successful; hence, threshold values for higher members of these series refer to mixtures rather than to single compounds.

RESULTS

Three homologous series were studied, the methylene, ethylene, and propylene glycols. The first series was represented by ethylene glycol, trimethylene glycol, pentanediol-1,5, hexanediol-1,6, and decanediol-1,10. Since no homologous 7, 8, or 9 carbon diols were obtainable, 2-ethyl hexanediol-1,3, and octanediol-2,3 were substituted to give points of reference between C_6 and C_{10} . The second series was represented by ethylene glycol through polyethylene glycol 6000; the third, by ethylene, propylene, dipropylene, and tripropylene glycols and by three polypropylene glycols with average molecular weights of 400, 750, and 1200. The data for the glycols are summarized in the following equations, which describe the regression of log concentration (Y) rejected by 50 per cent of the flies on log No. of carbon atoms (X). The number of carbon atoms was chosen as a characteristic because complete data for other properties, *e.g.* boiling point and solubility, were not available. In making these calculations the points for the individual compounds have been given equal values irrespective of the number of measurements by which each was determined. Data for the corresponding alcohols are given for comparison.

1. Alcohols through 1-butanol

$$Y = 0.22825 - 1.7573(X - 0.345)$$

$$\text{variance of } a = 0.0088; \text{variance of } b = 0.0592$$

2. Alcohols from butanol to 1-octanol, inclusive
 $Y = -2.552 - 13.5152(X - -0.80625)$
 variance of $a = 0.0023$; variance of $b = 0.3247$
3. Diols through hexanediol-1,6
 $Y = 0.35175 - 2.3040(X - 0.56375)$
 variance of $a = 0.0068$; variance of $b = 0.1938$
4. Hexanediol-1,6 to decamethylene glycol
 $Y = -1.181 - 9.8900(X - 0.88908)$
 curve based on only two points
5. Polyethylene glycols, pure compounds
 $Y = 0.39175 - 1.8556(X - 0.646)$
 variance of $a = 0.0016$; variance of $b = 0.0303$
6. Polyethylene glycol mixtures
 $Y = -0.1900 - 0.5359(X - 1.35467)$
 variance of $a = 0.0002$; variance of $b = 0.0016$
7. Ethylene glycol through dipropylene glycol
 $Y = 0.5323 - 2.4592(X - 0.5187)$
 variance of $a = 0$; variance of $b = 0$.
8. Polypropylene glycol mixtures
 $Y = -2.5370 - 4.2642(X - 1.4533)$
 variance of $a = 0.0144$; variance of $b = 0.3407$

The type formula for the above is $Y = a + b(X - \bar{x})$, where a has the numerical value of \bar{y} and where \bar{x} and \bar{y} are the mean values of empirical X and Y respectively.

Results for the glycols are summarized in Table I, and for the first eight primary normal alcohols in Table II.

DISCUSSION

(a) *The Distribution of Acceptance and Rejection Thresholds in Insect Populations.*—An acceptance or rejection threshold may be defined as the least concentration of a chemical required to cause (or prevent) the manifestation of some response selected by the investigator and interpreted as acceptance or refusal. Despite precautions taken in the determination it is commonly observed that not all individuals of a given species respond alike to a single concentration of the test agent. Over a certain critical range, at least, some specimens will accept while others reject; and although with a small group of individuals it is possible usually to extend the range in both directions (unless solubility interferes) until 100 per cent acceptance or refusal is obtained, increasing the number of insects sampled generally requires a further extension of range in order to achieve 100 per cent response. These facts raise questions as to the accuracy and significance of threshold determinations, and as to the most suitable procedures for their measurement.

In studying with *Phormia* the rejection of a large number of unacceptable compounds, a definite relationship between concentration of the test material and the distribution of thresholds has been noted regularly in samples of flies selected at random from a population of known age which had been reared

under standard conditions. For such samples, the scattering of thresholds is, within the limits of experimental error, normal with respect to the logarithm of

TABLE I
*Response of *Phormia* to Glycols in 0.1 M Sucrose*

Glycol	No. of C atoms	Log molar concentration rejected by 50 per cent ± 2.375 s.e.	$a \pm \text{s.e.}^*$	$b \pm \text{s.e.}^*$	\bar{x}^*	No. of flies tested
Ethylene	2	1.076 ± 0.046	4.981 ± 0.087	4.858 ± 0.590	1.072	254
Trimethylene	3	0.359 ± 0.151	5.188 ± 0.182	3.144 ± 0.481	0.419	100
Pentanediol-1,5	5	0.056 ± 0.146	5.070 ± 0.154	2.736 ± 0.422	0.030	100
Hexanediol-1,6	6	-0.084 ± 0.129	4.996 ± 0.148	2.969 ± 0.393	-0.083	100
2-Ethyl hexanediol-1,3	8	-1.654 ± 0.141	5.019 ± 0.209	3.785 ± 0.693	-1.649	100
Octanediol-2,3	8	-1.884 ± 0.103	4.849 ± 0.230	2.278 ± 0.661	-1.950	100
Decanediol-1,10	10	$-2.278 \dagger$	—	—	—	100
Diethylene	4	0.374 ± 0.157	5.480 ± 0.171	3.069 ± 0.494	0.531	340
Triethylene	6	0.171 ± 0.119	5.346 ± 0.138	3.126 ± 0.378	0.282	144
Tetraethylene	8	-0.054 ± 0.101	4.989 ± 0.142	3.625 ± 0.480	-0.056	171
Propylene	3	0.621 ± 0.228	5.502 ± 0.375	5.010 ± 2.367	0.721	123
Dipropylene	6	-0.100 ± 0.148	5.172 ± 0.137	2.648 ± 0.321	-0.035	150
Tripropylene	9	-0.799 ± 0.109	5.231 ± 0.107	2.590 ± 0.386	-0.710	175
Polypropylene 400	13.5§	-1.235 ± 0.493	5.095 ± 0.532	2.797 ± 1.704	-1.201	100
Polypropylene 750	31.5§	-2.528 ± 0.193	5.377 ± 0.165	2.443 ± 0.525	-2.374	100
Polypropylene 1200	54§	-3.848 ± 0.352	4.903 ± 0.133	0.996 ± 0.328	-3.946	100
Polyethylene 200	8§	0.024 ± 0.178	5.314 ± 0.133	2.110 ± 0.409	0.173	100
Polyethylene 300	12§	-0.008 ± 0.094	5.407 ± 0.128	3.827 ± 0.524	0.098	152
Polyethylene 400	18§	-0.117 ± 0.336	5.431 ± 0.280	2.701 ± 1.341	0.042	100
Polyethylene 600	26§	-0.255 ± 0.266	5.282 ± 0.320	3.301 ± 1.386	-0.170	118
Polyethylene 1000	44§	-0.327 ± 0.119	5.041 ± 0.257	5.548 ± 1.642	-0.320	170
Polyethylene 1540	68§	-0.456 ± 0.073	4.808 ± 0.138	5.028 ± 0.934	-0.494	109
Polyethylene 4000	182§	Accepted in saturated solution				100
Polyethylene 6000	272§	Accepted in saturated solution				100

* The 4th, 5th, and 6th columns of the table give the calculated values for a , b , and \bar{x} in the equation $Y = a + b(X - \bar{x})$, which is the regression of per cent flies rejecting, Y , expressed as probits, on log concentration, X . s.e. = standard error.

† Estimated from 6 per cent rejection of the saturated solution (0.0016 M), by assuming a value of 3 for b .

§ Average number of carbon atoms.

concentration. The same sort of relationship is apparent also in the results of other entomologists who have reported sufficient numbers of tests to permit a comparable analysis. As evidence we present examples recalculated from the

work of Weis (21) with the butterfly, *Pyrameis*; von Frisch (10) with bees; and Frings (8, 9) with the American roach (Fig. 1). We are indebted to Dr. Frings for his kindness in arranging and making available his original data. Our own data for various glycols are shown in Figs. 2 and 3.

Recognition of this relationship has served as a useful guide in planning experiments and in the treatment of results. The procedure we have followed is essentially that advocated by Bliss (1) for dealing with dosage-mortality data. Separate samples of 1 to 3 day old flies taken at random from the culture are tested at each of six or seven concentrations within the critical range, and the percentages accepting or rejecting converted into probability units. A plot of these against log concentration yields a linear regression. The slope and position of the regression line, and the most probable value of the concentration

TABLE II
Response of Phormia to Normal Alcohols in 0.1 M Sucrose

Alcohol	No. of C atoms	Log molar concentration rejected by 50 per cent ± 2.575 S.E.	$a \pm \text{S.E.}$	$b \pm \text{S.E.}$	\bar{x}	No. of flies tested
Methanol	1	0.782 ± 0.205	4.928 ± 0.223	2.809 ± 0.662	0.757	125
Ethanol	2	0.377 ± 0.152	5.179 ± 0.248	4.304 ± 1.254	0.418	120
1-Propanol	3	0.077 ± 0.048	5.064 ± 0.170	9.076 ± 1.799	0.084	88
1-Butanol	4	-0.323 ± 0.066	5.363 ± 0.167	7.059 ± 1.348	-0.212	146
1-Pentanol	5	-1.122 ± 0.066	4.919 ± 0.191	7.507 ± 1.272	-1.132	105
1-Hexanol	6	-2.211 ± 0.136	4.991 ± 0.145	2.742 ± 0.491	-2.213	100
1-Heptanol	7	-2.935 ± 0.189	4.848 ± 0.142	1.968 ± 0.370	-3.012	100
1-Octanol	8	-3.940 ± 0.161	4.832 ± 0.150	2.445 ± 0.414	-4.008	100

required for 50 per cent response (or for some other chosen level), together with their variances, are calculated by the usual statistical methods.

Since the distribution of thresholds is normal with reference to log concentration, the use of the arithmetic mean for purposes of comparison (as in our earlier papers) is invalid. Fortunately, if similar numbers and similar series of concentrations have been used in the experiments, the arithmetic means will stand in about the same order relative to one another as the geometric means, so that the same qualitative conclusions are reached in both cases.

The treatment here adopted, in addition to providing a quantitatively more accurate measure of the response of a population, has the advantage that it permits the inclusion in comparisons of data on compounds which, on account of low solubility or weak stimulating power, cannot be investigated over the full range required for 100 per cent response. This is possible because the slope and position of the regression line can still be determined within known limits of accuracy on the basis of the results available from the range open to

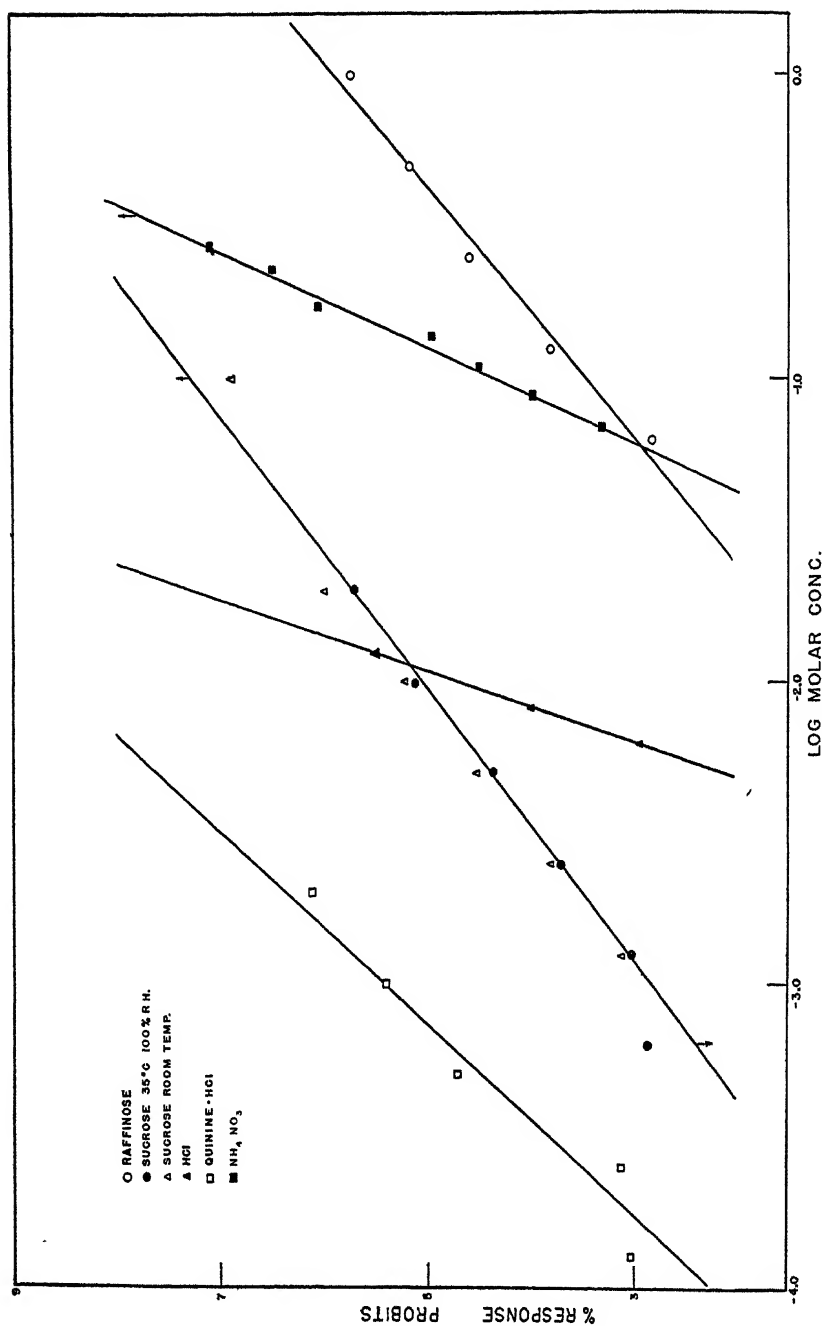


FIG. 1. Distribution of acceptance and rejection thresholds for various insects as a function of concentration. Raffinose—acceptance by *Pyrausta atalanta* L., from data of Weis (21); sucrose—acceptance by *Pyrausta atalanta* L., from data of Weis (21); HCl—rejection by *Apis mellifera* L., from data of von Frisch (10); quinine-HCl—rejection by *Apis mellifera* L., from data of von Frisch (10); NH₄NO₃—rejection by *Periplaneta americana* L., from data of Frings (8).

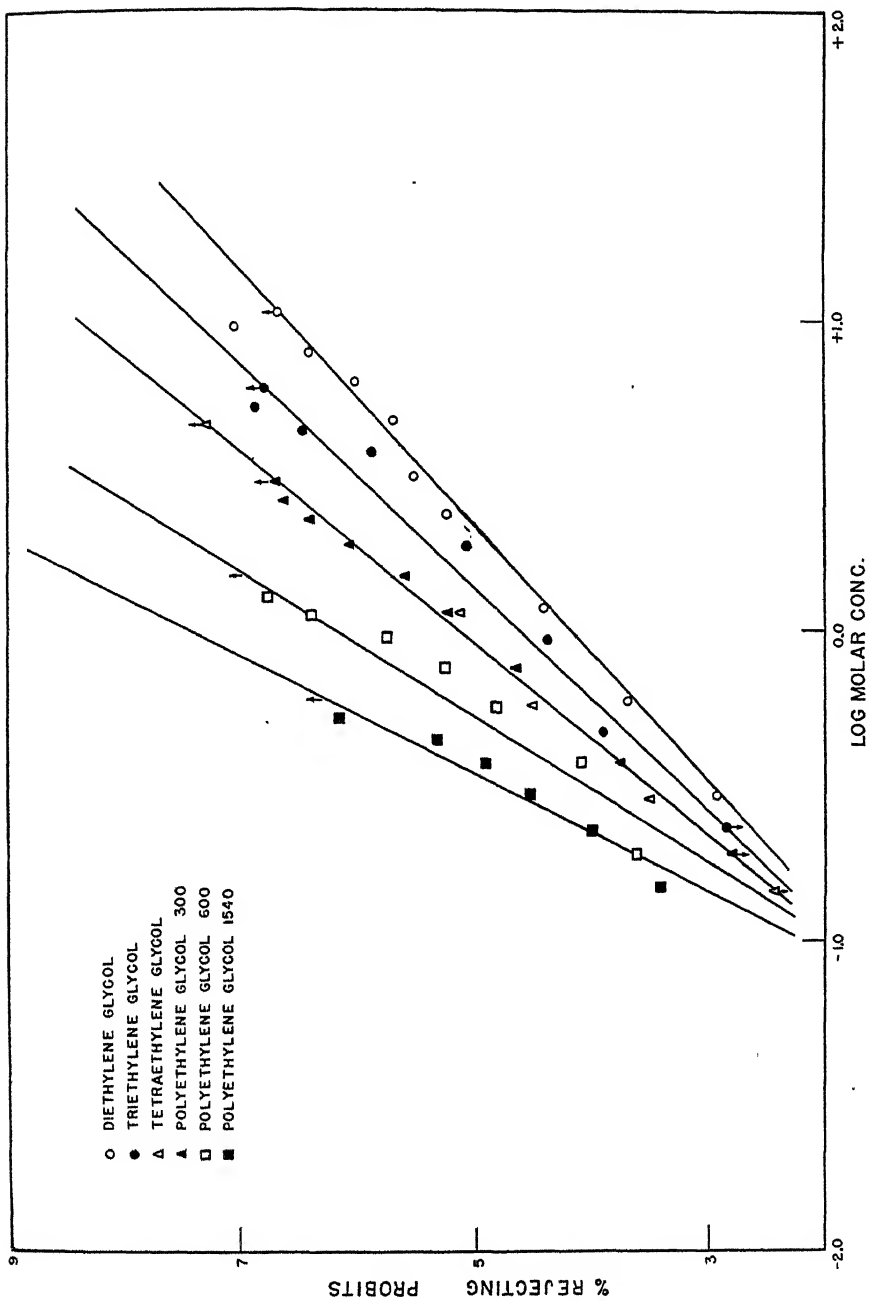


FIG. 2. Distribution of rejection thresholds for *Pharmia* for various glycols, as a function of concentration.

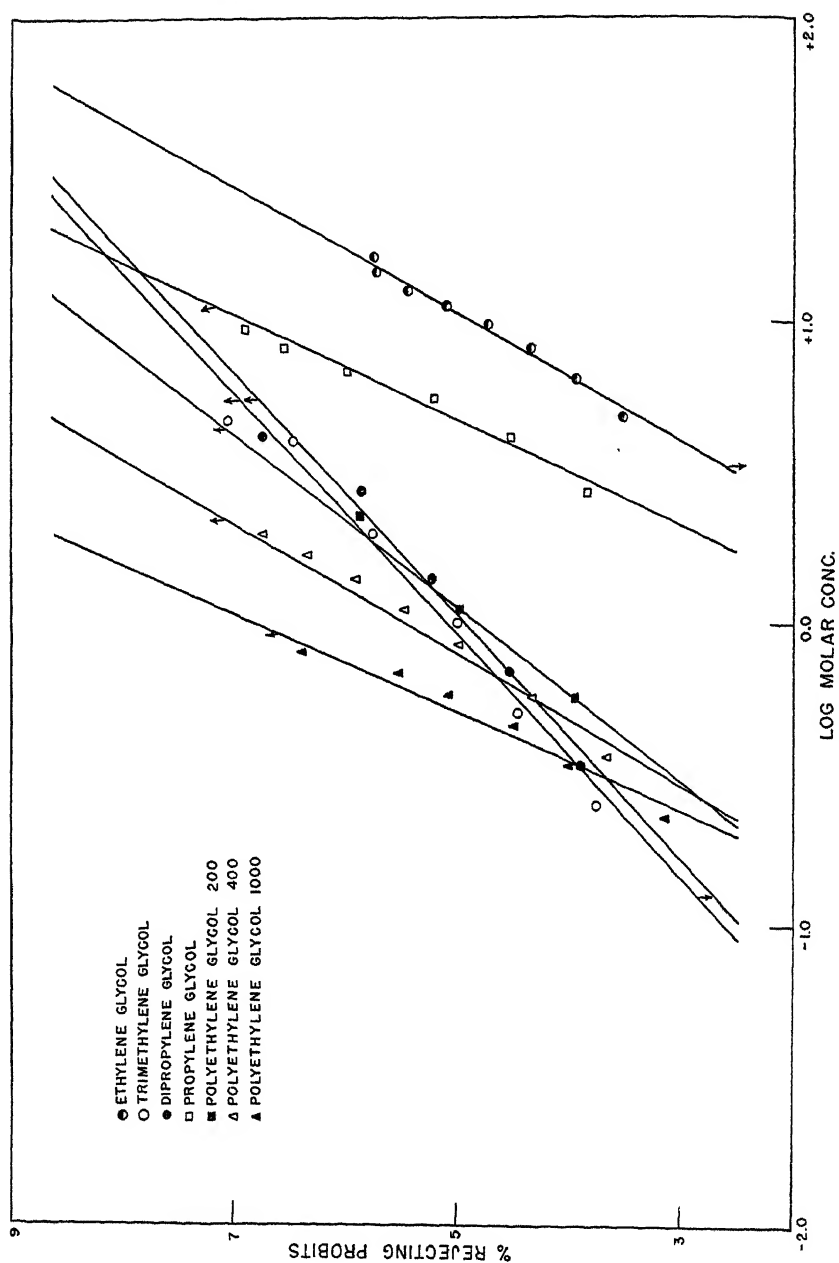


FIG. 3. Distribution of rejection thresholds for *Pharmia* for various glycols, as a function of concentration.

experimentation. Thus, for example, in our tests with ethylene glycol about one-quarter of the flies regularly accepted the undiluted compound containing 0.1 M sucrose, but this fact has not interfered with the calculation of the most probable value for the concentration rejected by 50 per cent of the population.

It is apparent from the literature on insect chemoreception that others must also have been aware that the rate of response is related to the logarithm of concentration, although we have found no definite statements to that effect. For instance, Eger (5) has recorded his results with lepidopterous larvae on a semilogarithmic scale, with percentages of rejection plotted against class units which are proportional to the logarithm of concentration, and has tacitly assumed a normal distribution by calculating the geometric means and their standard errors. Frings and O'Neal (9) expressed their data with *Tabanus* as geometric means which were determined by a method of graphical interpolation. That the situation is not peculiar to insects is indicated by the results of Krinner (12) with the minnow *Phoxinus*.

The underlying reason for the phenomenon described is not known. It is quite usual for the intensity of various expressions of a sensory response to be related approximately logarithmically to the intensity of the stimulus (here, concentration), and we have cited elsewhere (4) a number of examples in chemoreception where this appears to be the case. But at present we do not have any information on questions such as how the frequency of discharge of a single chemoreceptor and the number of units responding are dependent on stimulus intensity, nor can we judge whether these or yet other factors are at the base of differences between individual specimens.

(b) *The Relative Stimulating Effectiveness of the Glycols.*—For all series of homologous aliphatic compounds studied thus far it has been found that members of the series were rejected at logarithmically decreasing concentrations as the carbon chain increased in length. In every case only chains of CH_2 groups were involved. In the series represented by polyethylene and polypropylene glycols a different type of molecular chain than heretofore tested is formed by the presence of ether linkages. Members of the former, for example, are compounds of the general formula $\text{OHCH}_2(\text{CH}_2\text{OCH}_2)_x\text{CH}_2\text{OH}$. Nevertheless, these glycols as well as the straight diols conform to the previously established pattern (Fig. 4). In terms of molecular weight or number of carbon atoms the latter are slightly more stimulating than the ether-linked chains although the differences in stimulating effectiveness between the lower members of the three series are not statistically significant when the slopes of the lines of best fit are compared. The propylene series, members of which contain longer carbon chain links between oxygen atoms than do the polyethylene glycols, more nearly resemble the straight diols in stimulating power. Unfortunately physical data are not available for a complete analysis of this problem. It has been shown by Sauter (15, 16) and Fuller and Frosch (11) that the presence of ether or

glycol linkages in a chain causes distortion of the usual zigzag form with the consequent assumption of a helical form. Inasmuch as chain length appears to be of importance as far as stimulation is concerned, it is not improbable that an explanation of the differences between straight diols and polyethylene or polypropylene compounds may be associated with the difference in molecular shape.

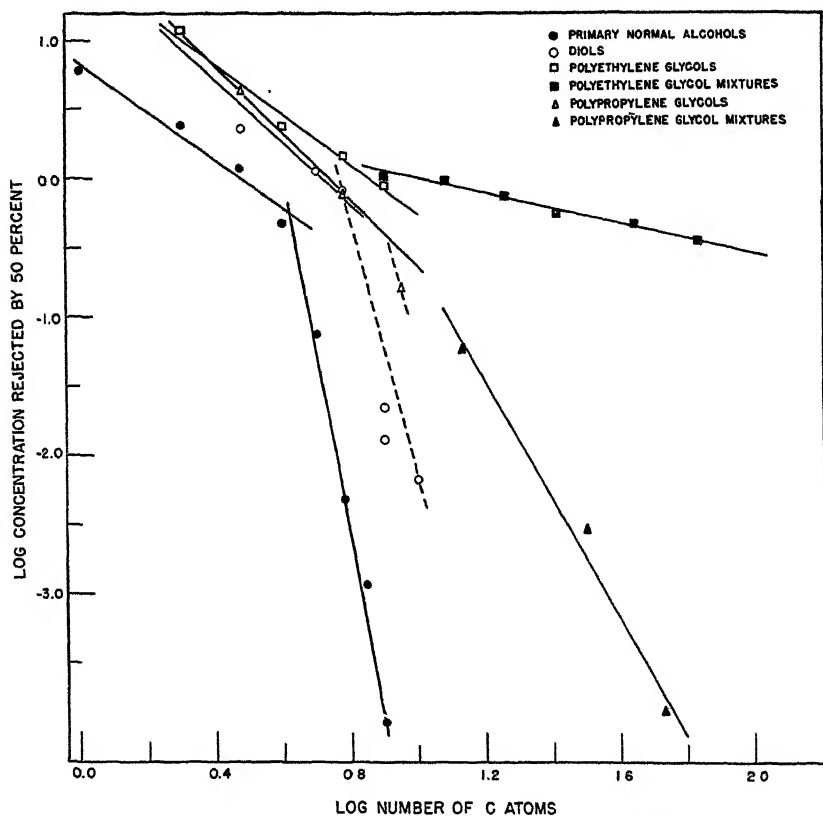


FIG. 4. Rejection of glycols and alcohols by *Phormia*.

At that point in the ethylene series where polymer mixtures were employed in lieu of pure compounds, which were unobtainable, there is a significant change in stimulating effectiveness. The mixtures are less stimulating than would be expected from their average molecular weight. The difference in slope of the pure and mixed members is 1.3197, about $7.4 \times$ its standard error of 0.1786. A comparable relationship probably exists between the pure polypropylene glycols and mixed members. At the moment we have no satisfactory explanation of this phenomenon. Lovell and Hibbert (14) have stated that uni-

polymers may show outstanding differences in properties from a mixture of homologues with the same average molecular weight; however, no comparative data were given. Hence, while we can point to comparable physiological differences, we are unable to state whether the relation is direct or inverse.

Saturated solutions of polyethylene glycols 4000 and 6000 did not stimulate. Thus the highest effective member tested is polyethylene glycol 1540. The former solutions were 0.2 M and 0.1 M respectively. The effective concentrations, if the curve is extrapolated, fall above these values. Had the reverse been true, it would have indicated that the stimulative efficiencies of compounds at this point had begun to decrease. Data from other sources also militate against this possibility. Studies of the toxicity of single oral doses of glycol polymers to the rat, rabbit, and guinea pig (13, 17-19) have shown that the LD_{50} , if measured as molar concentration, decreases in a more or less regular manner with increasing molecular weight. In these studies it was possible to obtain a value for a polymer as high as 3600, and the curve shows no tendency to break at this point. It is noteworthy that toxicity data on mammals should parallel so closely rejection thresholds in insects. This may be construed as further evidence to support the idea that what is being measured in this and other cases is probably the rate of access of the compounds to the system rather than their final interaction with the processes under observation (*cf.* reference 3).

It is of further interest that such large molecules as 1540 should stimulate the chemical senses. Not many large ones appear to do so. Von Frisch (10) recorded that bees accepted with slight hesitation a 1 M sucrose solution containing 1/5 per cent colocynthin ($C_{56}H_{84}O_{23}$) and that eupatorin ($C_{42}H_{72}O_{21}$) is tasteless or slightly bitter. Some proteins, *e.g.* casein, hen's egg albumin, horse serum albumin, and hemoglobin, are reported by Thorpe *et al.* (20) as eliciting the biting response in wireworms.

When glycols are compared with the corresponding alcohols, it is seen that the introduction of a second hydroxy radical renders the compound much less stimulating. Thus the median rejection threshold for ethylene glycol is 11.91 M as compared with 2.38 M for ethanol, and for trimethylene, 2.287 M as compared with 1.195 M for *n*-propyl alcohol. The effect of —OH substitution here agrees with that observed by Chadwick and Dethier (2) with aliphatic acids. From a comparison based on the lines of best fit it is seen that the alcohols corresponding to the first three diols average about four times as stimulating as the latter. Similarly calculated, the alcohols corresponding to the diols from hexanediol-1,6 to decamethylene glycol are more than one hundred times as stimulating.

That the position of the hydroxy substitution is of some importance is observed when trimethylene and propylene glycols are compared. As would be expected from a comparison of the corresponding alcohols, the straight chain

compound is more stimulating than its branched isomer. Position effect has been studied in a large series of compounds and is to be treated at greater length in a subsequent communication now in preparation.

Present analyses indicate the presence of a break in the curve describing the stimulating effectiveness of the methylene series (*cf.* Fig. 4). Reexamination of our results with the primary normal alcohols also has shown the existence of a definite sharp break at or beyond *n*-butanol. The slope of the curve for the lower alcohols is -1.7573 ± 0.2433 (standard error); for the higher members it is -13.5152 ± 0.5698 . The line of best fit for the diols (ethylene glycol through hexanediol-1,6) has a slope of -2.3040 ± 0.4402 ; *i. e.*, not significantly different from that for the lower portion of the alcohol series. If the tentative threshold value given in Table I for decamethylene glycol is accepted, a line drawn between this point and that for hexanediol-1,6 has a slope of -9.8900 . Although we hesitate to stress this determination of the portion of the curve for the higher diols in view of the uncertainty of the threshold value for decamethylene glycol, the results found with 2-ethyl hexanediol-1,3 and octanediol-2,3 strengthen the case for such an interpretation. These C_8 diols are not strictly homologous with the other members of this series; however, when a comparison is made of isomers such as propylene and trimethylene glycols or various alcohols (3), the difference in stimulating effectiveness is not great unless the isomers differ markedly in such properties as solubility, boiling point, etc. With reference to the polypropylene glycols, not enough unipolymers are presently available for testing to decide whether a similar break may also occur in this series. While it is possible to calculate a single line of best fit for the entire group it is unlikely that this represents the true situation. There is a fair probability that the slope of such a line (-3.5028) is too great for the range below C_6 and that unipolymers with more than twelve carbon atoms might be significantly more stimulating than the corresponding mixtures.

SUMMARY

The rejection thresholds of *Phormia regina* Meigen for twenty-four glycols have been determined. A definite relationship between the concentration of the test material and the distribution of thresholds has been noted regularly in samples of flies selected at random from a population of known age which had been reared under standard conditions. The scattering of thresholds is normal with respect to the logarithm of concentration. Recalculation of the data of other workers reveals the same sort of relationship with other species of insects and the minnow *Phoxinus*. The underlying reason for the phenomenon is not known.

The glycols in common with other series of homologous aliphatic compounds are rejected at logarithmically decreasing concentrations as the chain length is

increased. In general the straight chain diols are more stimulating than the corresponding polyethylene and polypropylene glycols. This difference is related in some manner to the presence of ether linkages in the latter. Polypropylene glycols, with chains of three carbon atoms between the ether linkages are more stimulating than polyethylene glycols, where the spacing is —O—C—C—O— . Unipolymers are more stimulating than mixtures of homologues with the same average molecular weights. Polyethylene glycol 1540 is the largest molecule of measured molecular weight known to stimulate chemoreceptors. The introduction of a second terminal hydroxyl group into the straight hydrocarbon chain reduces the stimulating effect. Alcohols corresponding to the first three diols average about four times as stimulating as the latter while those corresponding to the higher diols average more than one hundred times as stimulating.

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PHYSICAL STRUCTURE OF SHELL MEMBRANES

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INTRODUCTION

For the metabolic processes involved in the development of the chick embryo, an adequate supply of respiratory gases is required. Several experimenters have shown that the air enters initially through small holes in the egg shell. Romanoff (1) studied, in addition, the permeability to air and other gases of the lamination formed from both the shell and shell membrane and found values of the order of several million times those obtained from high polymer membrane such as natural rubber. Moreover, he found that, generally speaking, the lighter gases were more penetrating than the heavy ones. These two facts, taken together, indicated that the gas transfer probably took place through small holes in *both* the shell and shell membrane. Since the presence of holes in the shell had been carefully demonstrated by Marshall and Cruikshank (2), the present authors thought it would be of interest to study the shell membrane alone. This membrane consists of two distinct layers which are everywhere in contact except at the rounded part of the egg where they separate to form an air sack (3). The results obtained show clearly that *dried* shell membranes do indeed contain very small holes and that a very rough estimate of their mean size may be obtained by gas permeability measurements alone. This estimate has been verified by electron microscope pictures of the shell membrane.

The Permeability Method

The transfer of gas through a film containing capillary pores may take place by true viscous flow, or by diffusion, or by a combination of these two. In any case, experiment and theory both show that the transfer rate *decreases* with increasing temperature. On the other hand, if no pores are present in the film, the transfer of gas will take place by interstitial diffusion of the activated type, the rate of which *increases* exponentially with the temperature. Our method of detecting the presence of pores in the shell membrane consisted simply in taking permeability measurements at two different temperatures for a series of common gases including H₂, He, N₂, O₂, and air. The permeability for all these gases showed a decrease with increasing temperature.

Experimental Arrangement and Procedure

The essential part of the experimental set-up is shown in Fig. 1. This whole permeability unit was placed in a water bath whose temperature was thermostatically controlled. Also in the water bath was placed a 10 foot coil of $\frac{1}{4}$ inch copper tubing

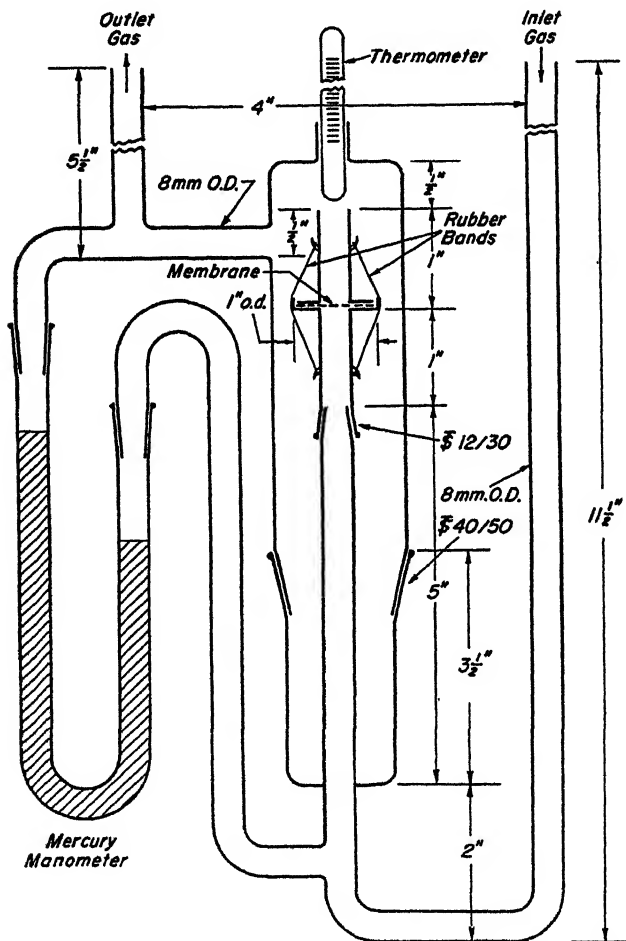


FIG. 1. The permeability unit

through which the gas was passed before entering the permeability unit. This arrangement insured that the temperature of the gas in contact with the membrane was the same as that of the bath.

The shell membrane was stripped from the shell and mounted between two ground-glass plates, as illustrated in Fig. 1. The subassembly containing the membrane was then placed in a desiccator and dried for at least 24 hours. In the drying process the membrane stuck firmly to the ground-glass plates, thereby forming a gas-tight seal.

The experimental procedure consisted simply of passing gas through the membrane under a known pressure difference and at a known temperature, and of determining the rate of passage of the gas by collecting it over water in a pneumatic trough. A series of gases difficultly soluble in water was first run through the membrane at room temperature and at fixed pressures. The temperature was then increased about 15° or 20°C., but the pressures were left unchanged and the procedure was repeated. To check the reproducibility of the data, some of the experiments were repeated. The membrane was checked for macroscopic fissures and cracks at the end of the experiments by saturating it with water. In this condition, an undamaged membrane was completely impervious to all gases.

Summary of the Findings

Different fresh embryonated hen egg shell membranes were subjected to permeability tests. In each case a circular area of 0.57 cm.² was exposed to

TABLE I

Permeability Data on Membrane 1

Membrane area = 0.57 cm.², membrane thickness = 0.0071 cm., barometric pressure = 74.5 cm. Hg, room temperature = 23.3°C.

Experiment No.	Gas used	Gas rate	Temperature	Pressure drop
		<i>cc./sec.</i>	<i>°C</i>	<i>cm. Hg</i>
1	O ₂	15.6	23	2.70
5	O ₂	15.2	23	2.70
2	N ₂	18.6	23	2.70
6	H ₂	42.6	23	2.70
4	O ₂	14.5	38	2.70
9	O ₂	14.6	38	2.70
3	N ₂	17.6	38	2.70
8	N ₂	17.5	38	2.70
7	H ₂	40.0	38	2.70

the gas stream. The thickness of the membranes varied slightly but all were close to 0.007 cm. The data are summarized in Tables I to III. The number designating the gas rate is in each case the average of from 5 to 10 separate determinations, the gas volume being measured to room temperature and pressure.

Interpretation of the Data

The data clearly show that the permeability of the shell membrane to gases decreases with increasing temperature. To draw the conclusion from this fact that the membranes contain micropores requires a closer look into the theory of the transfer of gas through small capillaries. This problem has been carefully studied by Sherwood (4) and his coworkers. They have demonstrated

TABLE II
Permeability Data on Membrane 2

Membrane area = 0.57 cm.², membrane thickness = 0.0065 cm., barometric pressure = 74.5 cm. Hg, room temperature = 23.0°C.

Experiment No.	Gas used	Gas rate	Temperature	Pressure drop
		<i>cc./sec.</i>	<i>°C.</i>	<i>cm. Hg</i>
10	N ₂	23.7	25	3.0
15	N ₂	23.8	25	3.0
19	N ₂	24.7	25	3.0
11	He	28.6	25	3.0
14	He	28.5	25	3.0
12	H ₂	50.2	25	3.0
15	H ₂	49.6	25	3.0
16	N ₂	21.8	45	3.0
17	He	26.1	45	3.0
18	H ₂	45.4	45	3.0

TABLE III
Permeability Data on Membrane 3

Membrane area = 0.57 cm.², membrane thickness = 0.0065 cm., barometric pressure = 74.7 cm. Hg, room temperature = 22.3°C.

Experiment No.	Gas used	Gas rate	Temperature	Pressure drop
		<i>cc./sec.</i>	<i>°C.</i>	<i>cm. Hg</i>
21	O ₂	26.7	24	3.0
25	O ₂	28.9	24	3.0
20	N ₂	37.9	24	3.0
24	N ₂	38.9	24	3.0
22	H ₂	89.4	24	3.0
23	Air	37.3	24	3.0
27	O ₂	26.4	37	3.0
31	O ₂	25.8	37	3.0
26	N ₂	36.6	37	3.0
30	N ₂	36.0	37	3.0
28	H ₂	84.5	37	3.0
29	Air	35.3	37	3.0

that the rate of transfer of gases through cylindrical capillaries may be expressed by the single equation

$$\dot{n} = \frac{\pi D^4 (p_1^2 - p_2^2)}{256 \mu L R T} \left[1 + 8 \left(\frac{2}{f} - 1 \right) \frac{\lambda_m}{D} \right], \quad (1)$$

where \dot{n} represents the number of moles of gas passing through the capillary per

unit time. In this equation λ_m is the mean free path of the gas molecules at the mean pressure, p_m , in the capillary, D is the diameter of the capillary, and f represents the fraction of gas molecules striking the capillary wall that are diffusely reflected. The value of f is usually in the neighborhood of unity (4). The other symbols have their customary meanings: μ is the coefficient of viscosity and is independent of the pressure but not of the temperature, L is the length of the capillary, $(p_1 - p_2)$ the pressure drop across it, and T is the temperature. For purposes of calculation it is convenient to write the mean free path λ_m in terms of observable quantities. This may be done with the aid of kinetic theory, which gives

$$\lambda_m = \frac{\mu}{p_m} \left(\frac{\pi RT}{2M} \right)^{\frac{1}{2}}. \quad (2)$$

If this value for λ_m is substituted in equation (1), and if, in addition, equation (1) is separated into two terms, there results

$$\dot{n} = \frac{\pi D^4 (p_1^2 - p_2^2)}{256 \mu L R T} + \frac{\pi^2 D^3 (p_1 - p_2)}{16 L \sqrt{2 \pi M R T}} \left(\frac{2}{f} - 1 \right). \quad (3)$$

Now for a given capillary and fixed values of p_1 , p_2 , and T , the first term on the right-hand side depends only on the viscosity of the flowing gas, whereas the second term is independent of the viscosity and varies inversely as the square root of the molecular weight of the flowing gas. In other words, the first term on the right-hand side of equation (3) represents the transfer of gas by viscous flow, whereas the second term represents the transfer of gas by diffusive flow according to Graham's law of diffusion. The fact which is of singular importance for the present considerations is that both terms in equation (3) decrease with increasing temperature, and, although in any actual membrane we will almost certainly not be dealing with uniform cylindrical capillaries, the pressure and temperature dependence of the flow rate should be given by this equation. We choose, therefore, to write equation (3) in the form

$$= \frac{a}{\mu T} + \frac{b}{\sqrt{MT}} \left(\frac{2}{f} - 1 \right), \quad (4)$$

where a and b are constants depending on the geometry of the capillary and on the pressures p_1 and p_2 . For a membrane containing N capillaries per square centimeter, of arbitrary size and shape, there will be N equations similar to equation (4), so that

$$\sum_{i=1}^N \dot{n}_i = \frac{1}{\mu T} \sum_{i=1}^N a_i + \frac{1}{\sqrt{MT}} \left(\frac{2}{f} - 1 \right) \sum_{i=1}^N b_i. \quad (5)$$

The sum on the left hand is related to the volume, \dot{V} , of gas passing through

the membrane per unit time per *unit area* measured at room temperature T' and atmospheric pressure p' , by the relation

$$\sum_{i=1}^N \dot{n}_i = \left(\frac{p'}{RT'} \dot{V} \right). \quad (6)$$

Equation (5) may, therefore, finally be written in the form

$$\dot{V} = \frac{A}{\mu T} + \frac{B}{\sqrt{MT}} \left(\frac{2}{f} - 1 \right), \quad (7)$$

where

$$A = \frac{RT'}{p'} \sum_{i=1}^N a_i, \quad (8)$$

and

$$B = \frac{RT'}{p'} \sum_{i=1}^N b_i. \quad (9)$$

Equation (7) not only clearly shows that the permeability of a membrane containing micropores decreases with increasing temperature, but it also provides a method for estimating the mean equivalent diameter of the pores. The method of carrying out the calculation is described in detail in the next section.*

Estimation of Pore Diameter

For a membrane comprised of a system of N identical cylindrical pores per square centimeter, the expression for A in equation (8) becomes

$$A = \frac{NT'}{p'} \left[\frac{\pi D^4 (p_1 - p_2) p_m}{128L} \right] \quad (10)$$

and the expression for B in equation (9) becomes

$$B = \frac{NT'}{p'} \left[\frac{\pi^2 D^3 (p_1 - p_2)}{16L} \left(\frac{R}{2\pi} \right)^{\frac{1}{2}} \right]. \quad (11)$$

The ratio of B to A is then simply

$$\frac{B}{A} = \frac{4\sqrt{2\pi R}}{D p_m},$$

and hence the diameter D is given by

$$D = \frac{4A}{B} \frac{\sqrt{2\pi R}}{p_m}. \quad (12)$$

*A similar procedure has been used by H. Adzumi, *Bull. Chem. Soc. Japan*, 1937, **12**, 304.

Now if A/B is determined by experiment, D may be calculated. To evaluate A/B we write equation (7) in the form

$$\mu T \dot{V} = \mu \sqrt{T/\bar{M}} B \left(\frac{2}{f} - 1 \right) + A. \quad (13)$$

If then the group $\mu T \dot{V}$ is plotted against the group $\mu \sqrt{T/\bar{M}}$ for all the gases used with a given shell membrane, a straight line should be obtained, provided f is constant (5). The slope of this line will be the quantity $B \left(\frac{2}{f} - 1 \right)$ and the intercept on the vertical axis the quantity A . On Fig. 2, three such curves are shown, one for each shell membrane. All the curves are straight lines, indicating that f is, in fact, a constant within experimental error.

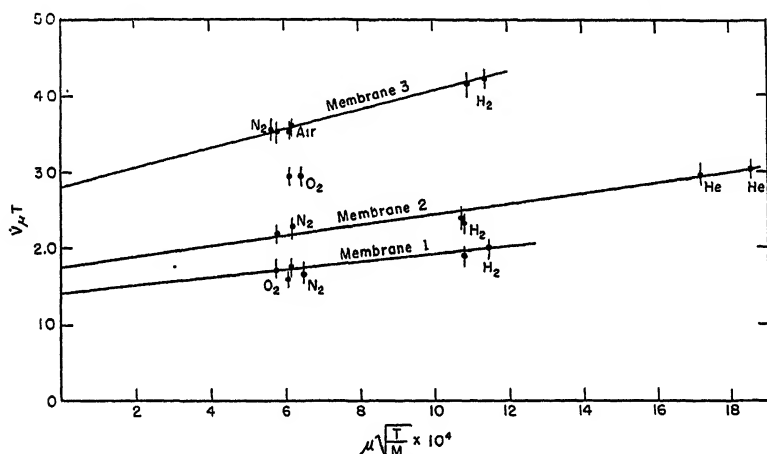


FIG. 2. Experimental verification of fluid flow law

On the assumption that $f = 1$, D has been calculated for each shell membrane using equation (12). The values 2.4, 2.3, and 2.0 microns have been obtained for shell membranes 1, 2, and 3 respectively. If the pores are not cylindrical, these numbers must be looked upon as equivalent mean diameters. Alternatively, one could use the assumption that the cross-section of each pore is a square and carry out a similar calculation.

We may now go back to equation (11) and calculate the surface density of pores, N . In doing so, we are obliged to use the third power of the diameter, D , which is only roughly known, and, therefore, the calculated results may be expected to be correct in order of magnitude only. The calculated values for the surface density were 10×10^6 , 15×10^6 , and 40×10^6 per sq. cm. for shell membranes 1, 2, and 3, respectively. As will be seen below, these values are in good agreement with the electron micrograph data.

The ratio of the second term to the first term of equation (7) indicates the relative rate at which gas is transferred through the membrane by diffusive and viscous flow. If this relative rate is designated by the symbol G , we have

$$G = \frac{B}{A} \left(\frac{2}{f} - 1 \right) \mu \sqrt{T/M}. \quad (14)$$

This equation reveals in a very transparent way the manner in which this



FIG. 3. Electron micrograph of shell membrane

relative rate varies with the several experimental variables. In virtue of equation (2), it may be also be written in the form

$$G = \frac{8\lambda_m}{D} \left(\frac{2}{f} - 1 \right), \quad (15)$$

which discloses that the flow will be predominantly diffusive in character only where the ratio of the mean free path to the mean diameter of the pores is very large. In this situation the second term in equation (7) is the dominant one and Graham's law of diffusion will be accurately true. However, the ratio λ_m/D is seldom large at pressures in the neighborhood of 1 atmosphere, and a natural porous membrane obeying Graham's law would be an exceptional one indeed.

The Electron Micrographs

After the above permeability study had been completed, it occurred to the authors that the electron microscope might supply direct evidence of the existence of micropores. Through the kind cooperation of Dr. Harold C. O'Brien of the University of Pittsburgh, we obtained electron micrographs of three different parts of a dried shell membrane. One of these is presented in Fig. 3. The picture is that of an actual dried shell membrane and not of a plastic replica. The white areas designate the holes in the membrane, and, while they vary considerably in size, one can deduce from the scale line that they are actually in the neighborhood of 1 micron. The area covered by each electron micrograph was about 150×10^{-8} sq. cm. and the number of holes in each picture was about 30. Hence the surface density of holes, N , was about twenty millions.

One can deduce from the electron micrographs that the holes are gaps in a loosely woven fibrous lattice, the individual fibers being again of the order of 1 micron in diameter. The fibers have been shown from chemical studies to consist of a pure typical keratin (6).

CONCLUSIONS

The results obtained in the present work bring out clearly that the dried shell membrane is an open lattice-like network containing micropores of the order of 1 micron in mean equivalent diameter and whose surface density is roughly 20 millions per sq. cm. The gas transfer through this network is largely by viscous flow and accordingly does not obey Graham's law of diffusion.

The data make clear that dried shell membranes are extremely permeable to all the gases examined and that wet membranes are completely impermeable. This fact leads one to suspect that during embryonic development, the rate of influx of gases is controlled in some measure by the moisture content of the shell membrane.

The permeability technique developed here should be of value in the study of other membranes and especially natural membranes of biological importance.

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REGULATORY MECHANISMS OF CELLULAR RESPIRATION

I. THE RÔLE OF CELL MEMBRANES: URANIUM INHIBITION OF CELLULAR RESPIRATION

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The large number of inhibitors of cellular respiration hitherto described have been reported to act by inhibiting the activity of enzyme systems. They do so (1) by combining with the activating protein, either through some groups essential for activity (for example, the —SH groups), through denaturation of the molecule, or by combination on the side chains where substrates or prosthetic groups form the protein-substrate complex (structural inhibitors); (2) by combining with the prosthetic groups of enzymes (diphosphothiamine, pyridoxal, pantothenic acid, etc.); (3) by combining with the series of oxidation reduction systems (pyridine nucleotides, flavins, cytochromes) which transfer electrons from oxidizable substrate to molecular oxygen. Besides this direct action on the components of enzyme systems, cellular respiration may be affected by alteration of the varied mechanisms which regulate in the living cells the rate and the direction of enzymatic reactions. One of these regulating mechanisms is the state of the cellular membrane. It is generally agreed that the cell membrane is a lipid-protein system possessing varying degrees of permeability where penetration occurs by passage through the pores of the membrane or through solution in the lipid portion. Any alteration of the solubility coefficient in the lipid phase or changes of the pore size will bring forth alterations in the rate of passage of substrates, and as a consequence alterations in the metabolism of the cell. We present in this paper experiments on the inhibition of cellular oxidations produced by uranyl nitrate, which have been interpreted as being due to combination of uranium with the protein layer of the cell membrane, bringing thus an increased impermeability to the passage of certain oxidizable substrates.

EXPERIMENTAL

The yeast cells used in these experiments were brewers' yeast from Keely Brewing Company, Chicago, and bakers' yeast from Fleischmann. The first fermented

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glucose to CO_2 and alcohol, but did not consume oxygen in air. Bakers' yeast, on the other hand, not only fermented glucose anaerobically but oxidized with great speed glucose, ethyl alcohol, and acetate. The cake of bakers' yeast (10 gm.) was washed twice with 250 cc. of distilled water and was aerated with oxygen for 20 hours at room temperature (25–26°). It was then centrifuged and suspended again in distilled water. Such yeast cells had little endogenous respiration (approximately 2 to 4 c.mm. O_2 per mg. dry weight per hour) and remained with unaltered metabolic activities for 7 days when kept at 3°. Gonococci were obtained from Dr. Phillip C. Miller's laboratory and *E. coli* from Miss Helen Van Sant's laboratory of the Department of Medicine. *Ps. aeruginosa*, *M. lysodeikticus*, and *M. creatinovorans* were obtained from the National Type Culture Collection, Georgetown University. Crystalline plasma albumin was obtained from Armour Company. The ultrafiltration experiments as well as the determination of uranium were made according to the methods described by Muntz and Barron (1). Nitrogen determinations were made by the micro-Kjeldahl method of Ma and Zuazaga (2), glucose by the method of Nelson (3). The cell-free brewers' yeast juice was prepared according to Nilsson and Alms (4). The clear, golden-brown, supernatant fluid fermented hexosediphosphate when glucose was present, maximum CO_2 production occurring when the substrates were added at a ratio of 4 moles of glucose per mole of hexosediphosphate. Hexosediphosphate or glucose alone was not fermented; apparently this preparation lacks the enzyme apyrase, which according to Meyerhof (5) is easily inactivated by the maceration procedure. The yeast juice prepared in this manner is 0.1 M in inorganic P so that no additional phosphate need be added in order to obtain maximum activity. Work with sea urchin (*Arbacia punctulata*) sperm was performed at the Marine Biological Laboratory, Woods Hole. Shed sperm were centrifuged for 5 minutes, the supernatant coelomic fluid was pipetted off, and the sperm were suspended in about 20 volumes of Ca-free artificial sea water. To this was added an equal volume of 0.1 M acetate buffer in artificial sea water (pH 6.5). The pH of the final suspension was then adjusted to give a pH value of 6.3 to 6.6. $\text{UO}_2(\text{NO}_3)_2$ was made up fresh just before use, in sea water-acetate buffer, pH 6.5. Dry weights of the sea urchin sperm suspension were determined as follows: 0.8 cc. of the suspension was centrifuged for 5 minutes in an air-driven high speed centrifuge at 75 pounds air pressure (16,000 g); the supernatant fluid was removed, and 0.7 cc. of the same suspension was added to the tube. This was centrifuged again for 10 minutes at the same 75 pounds air pressure. The supernatant fluid was removed as completely as possible, and the tubes were dried at 105° overnight. Dry weights of sperm were quite uniform, 18 to 20 per cent of the fresh weight.

Uranium and Proteins.—It is well known that uranyl nitrate, like most salts of heavy metals, precipitates proteins. In small amounts, however, U combines reversibly with proteins, as was shown by Muntz and Barron (1) in their studies on the combination of serum proteins with uranium. The U-protein complex, formed mostly with the albumin fraction, was split on addition of bicarbonate. The combination of plasma crystalline albumin with uranium and its reversibility was studied by the ultrafiltration technique of Muntz and

Barron (1) at two pH values: 7.3 and 3.8. At pH 7.3, veronal buffer, addition of uranyl nitrate (0.001 M) to the buffer produced aggregates distinguished by the formation of a Tyndall effect. When albumin was present there was no Tyndall effect on addition of uranyl nitrate, an indication that the uranium had combined with protein. On ultrafiltration, 27 per cent of U passed through the membrane in the uranyl nitrate—buffer system, and 16 per cent in the uranyl nitrate—buffer—albumin system. Almost all of the U became ultrafilterable on addition of citrate at a ratio of U:citrate of 1:10 (Table I). The combination of U with albumin was not reversed on addition of phosphate at the same ratio as that of uranium and phosphate. This was demonstrated by adding phosphate to the albumin—buffer—uranium mixture. No precipitation

TABLE I

The Combination of Uranium with Crystalline Plasma Albumin. Effect of pH on the Reversibility of the Reaction. pH 7.3

In all experiments 3 cc. of 0.001 M $\text{UO}_2(\text{NO}_3)_2$ to 10 cc. volume. Veronal buffer, 0.05 M, pH 7.3. (1) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 7 cc. 0.154 M NaCl not ultrafiltered; (2) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 4 cc. NaCl; (3) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 250 mg. albumin + 4 cc. NaCl; (4) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 250 mg. albumin + 0.3 cc. citrate (0.1 M) + 3.7 cc. NaCl. Ultrafiltration for 1 hour. Uranium analysis in 1 cc. of ultrafiltrate. Figures give $\log I_0/I$ values = E .

Experiment No.	E values	Uranium in ultrafiltrate
		per cent
1. Control.....	0.660	100
2. U in buffer.....	0.180	27.3
3. U + buffer + albumin.....	0.108	16.4
4. U + buffer + albumin + citrate.....	0.610	92.4

of uranyl phosphate appeared after centrifugation for 1 hour at 3,000 R.P.M. In absence of protein, the UO_2HPO_4 precipitate appeared immediately.

At pH 3.8, in hippurate buffer, the albumin-U complex was more strongly associated than at pH 7.3, as shown by the ultrafiltration experiments in Table II. The uranium was not released from the protein on addition of citrate or phosphate. Uranyl nitrate in this buffer went through the membrane on ultrafiltration to the extent of 87 per cent. Further indication of the combination of uranium with albumin was found in the change of pH when uranyl nitrate was added to the protein. There was liberation of base and the solution became more alkaline.

Uranyl Nitrate and Enzymes.—The UO_2^{++} cation has a great tendency to form complex compounds with a large number of organic substances (6–8). Since the uranyl ion can form complexes with carboxyl, hydroxyl, keto, and amino groups, it is possible that it may form reversible complexes with re-

active groups in the side chains of the protein moiety of enzymes. Uranium may also inhibit enzyme reactions by combination with the prosthetic groups of enzymes or with adenosinetriphosphate, the high energy phosphate compound essential for energy transfer in a large number of oxidation-reductions. In fact, Singer *et al.* (9) found that uranium as uranyl nitrate inhibited reversibly a large number of enzyme systems, among which succinoxidase, hexosemono-phosphate oxidase, and lysozyme were most sensitive. Cytochrome oxidase, monoamine oxidase, pancreatic esterase, carboxypeptidase, cathepsine, and urease were partially inhibited. Hexokinase, pyruvate oxidase, and choline oxidase were slightly inhibited. Enzyme inhibition was reversible, and re-

TABLE II

The Combination of Uranium with Crystalline Plasma Albumin. Effect of pH on the Reversibility of the Reaction. pH 3.8

In all experiments 3 cc. of 0.001 M $\text{UO}_2(\text{NO}_3)_2$ to 10 cc. volume. Hippurate buffer, 0.05 M, pH 3.8. (1) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 7 cc. of 0.154 M NaCl not ultrafiltered; (2) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 4 cc. NaCl; (3) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 250 mg. albumin + 4 cc. NaCl; (4) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 250 mg. albumin + 3.7 cc. NaCl + 0.3 cc. phosphate (0.1 M) adjusted to pH 3.8; (5) 3 cc. $\text{UO}_2(\text{NO}_3)_2$ + 3 cc. buffer + 250 mg. albumin + 0.3 cc. citrate (0.1 M) adjusted to pH 3.8. Ultrafiltration for 1 hour. Uranium analysis in 1 cc. of ultrafiltrate. Figures give I_0/I values = E .

Experiment No.	E values	U in ultra-filtrate
		<i>per cent</i>
1. Control.....	0.690	100
2. U in buffer.....	0.600	87
3. U + albumin.....	0.130	18.8
4. U + albumin + phosphate....	0.125	18.1
5. U + albumin + citrate.....	0.145	21

activation was achieved on addition of citrate, α -hydroxyaspartate, oxalacetate, malate, tartrate, malonate, or oxalate. These substances reactivated the enzyme by the formation of U-complexes more strongly associated than the U-enzyme complex. Enzyme-U complex was not, however, dissociated on addition of phosphate. Furthermore, the degree of reactivation on addition of the organic acids above mentioned varied with the enzyme system. An example of such inhibition and reversal is given in Fig. 1, where the degree of inhibition of succinoxidase by varying concentrations of uranyl nitrate and reactivation by varying concentrations of citrate is given.

Uranyl Nitrate and Yeast Maceration Juice.—To test the effect of uranium on fermentation produced by cell-free yeast juice, the production of CO_2 at pH 6 was measured. Phosphate and veronal-acetate were used as buffers. MgCl_2 (0.004 M) and acetaldehyde (0.01 M) were added to glucose. The sub-

strate added to each vessel consisted of 0.1 cc. of 0.1 M hexosediphosphate and 0.2 cc. of 0.2 M glucose to a total of 3 cc. Uranyl nitrate inhibited the activity of yeast maceration juice, the degree of inhibition depending upon the

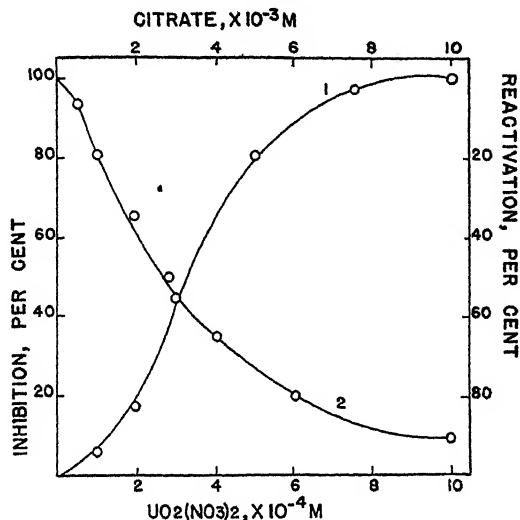


FIG. 1. Inhibition of succinoxidase (liver) by $UO_2(NO_3)_2$. Reactivation by Na citrate. (1) Inhibition of succinoxidase. (2) Reactivation of succinoxidase.

TABLE III

Inhibition by $UO_2(NO_3)_2$ of Yeast Juice Fermentation

Buffers, veronal-acetate, 0.032 M and phosphate, 0.01 M, pH 6.1. $UO_2(NO_3)_2$ brought to pH 6 before addition to the enzymes; final concentration, 0.001 M. Dry weight of yeast juice, 12.2 mg. per 0.1 cc. Temperature 23°. Final volume, 3 cc. Duration of experiments, 60 minutes.

Yeast juice	CO ₂ production			
	Veronal buffer		Phosphate buffer	
	Control	U	Control	U
cc.	c.mm.	c.mm.	c.mm.	c.mm.
0.05	93	5	39	4
0.1	242	16	119	9.5
0.2	462	236	478	250

amount of yeast juice treated with a given concentration of U (Table III). The inhibition was essentially the same whether the experiment was carried out in veronal-acetate or in phosphate buffer. To test the reactivation of the enzymes on addition of phosphate, experiments were performed with 0.1 cc. of juice to which $UO_2(NO_3)_2$ was added to give a final concentration of 1 X

10^{-3} M. After 10 minutes, increasing amounts of phosphate were added. Phosphate produced a partial reversal; however, 23 moles of phosphate was required per mole of uranium to produce 22 per cent reactivation (Table IV). It can be concluded from these experiments that uranium inhibition of fermentation enzymes in yeast juice is similar to other enzyme inhibitions in that phosphate does not readily reactivate the enzymes.

Uranium and Cellular Metabolism.—It has been shown that the fermentation of glucose by cell-free brewers' yeast juice was inhibited by uranium and that

TABLE IV

Inhibition by $\text{UO}_2(\text{NO}_3)_2$ of Yeast Juice Fermentation. Partial Reactivation with Phosphate

Conditions as in Table III. Buffer, veronal-acetate. Phosphate added 10 minutes after addition of $\text{UO}_2(\text{NO}_3)_2$.

Phosphate	CO ₂ production		
	Control	U	Inhibition
M	c.mm.	c.mm.	per cent
0.008	244	6.5	97
0.013	148	16	89
0.023	72	18	75

TABLE V

Inhibition by $\text{UO}_2(\text{NO}_3)_2$ of Glucose Fermentation in Brewers' Yeast Cells

Buffer, veronal-acetate, 0.05 M, pH 6.3. Gas phase, N_2 . Yeast suspension 0.3 cc. of 5 per cent cells. Duration of experiment, 60 minutes. Temperature 23°.

$\text{UO}_2(\text{NO}_3)_2$	Glucose utilization
M	mg.
0	6.2
1×10^{-3}	0
3×10^{-4}	0
1×10^{-4}	0

this inhibition was only partially released on addition of phosphate. When the same experiments were repeated with intact brewers' yeast cells, no inhibition was obtained when fermentation was measured manometrically in bicarbonate buffer (pH 7.3) and $\text{N}_2:\text{CO}_2$ as gas phase. This striking contrast between enzyme experiments with cell-free extracts and with living intact cells was found to be due to reactivation of the inhibition by bicarbonate. Indeed, when veronal was used as buffer (pH 6.3) and N_2 as gas phase, 1×10^{-4} M uranyl nitrate produced complete inhibition in the fermentation of glucose, as measured by its utilization (Table V). In these experiments the CO_2 formed was absorbed by KOH in the center cup of the Warburg vessels.

The oxidation of glucose by bakers' yeast was equally sensitive to the inhibitory action of uranium at both pH values, 7.3 and 3.8 (Table VI). The inhibition at pH 7.3 was completely released on addition of phosphate or bicarbonate; addition of phosphate at a ratio of P:U of 5:1 produced 85 per cent reactivation; even a ratio of 1:1 produced 73 per cent reactivation. Fur-

TABLE VI

Effect of pH on $UO_2(NO_3)_2$ Inhibition of Glucose Oxidation by Yeast

Concentration of buffer 0.025 M. Glucose, 0.01 M. $UO_2(NO_3)_2$, 2×10^{-5} M. Yeast, 3.7 mg. dry weight. Temperature 28°.

Buffer	pH	O ₂ uptake		Inhibition
		Control	$UO_2(NO_3)_2$	
		<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>
Veronal.....	7.3	257.5	23.5	91
Hippurate....	3.8	218.5	58.2	73.5

TABLE VII

Effect of pH on the Reversibility of $UO_2(NO_3)_2$ Inhibition of the Oxidation of Glucose by Bakers' Yeast

Buffer, 0.05 M. Glucose, 0.01 M. $UO_2(NO_3)_2$ 0.00002 M; citrate, or phosphate, 0.002 M; adenosinetriphosphate or hexosediphosphate, 0.001 M, added 10 minutes after the addition of $UO_2(NO_3)_2$ and adjusted to the pH of the buffer.

Additions	pH	O ₂ uptake		Inhibition
		Control	$UO_2(NO_3)_2$	
		<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>
Veronal.....	7.3	262	8.5	97
Veronal + phosphate.....	7.3	262	262	None
Veronal + citrate.....	7.3	263	263	None
Veronal + ATP.....	7.3	278	275	None
Veronal + hexose-diphosphate.....	7.3	280	278	None
Hippurate.....	3.8	218	58	73.5
Hippurate + phosphate.....	3.8	219	56.1	74
Hippurate + citrate.....	3.8	220	57	74
Hippurate + ATP.....	3.8	230	58	75

thermore, reactivation was achieved by adenosinetriphosphate, hexosediphosphate, citrate, and even by simply washing the cells with distilled water. However, inhibition by $UO_2(NO_3)_2$ at pH 3.8 (hippurate buffer) was not released on addition of phosphate, adenosinetriphosphate, or citrate (Table VII).

When the uranyl nitrate concentration was kept constant and the amount of yeast cells was varied, there was a close relationship between the inhibition of glucose oxidation and the ratio of yeast weight to U. Complete inhibition

was reached with 7.7 micrograms of uranium per mg. dry weight of yeast cells (Table VIII). An attempt was made to measure the amount of uranium adsorbed by yeast cells (2 mg. per cc.). Since a concentration of 2×10^{-5} M of uranyl nitrate inhibited 98 per cent glucose oxidation by 1.86 mg. of yeast, an analysis of uranium in the supernatant fluid would give the amount adsorbed by the cell at the saturation level. 102.6 mg. of yeast was suspended in 4.6 cc. H_2O . To this suspension was added 17.5 cc. 0.1 M veronal buffer, pH 7.3, and 10 cc. 2×10^{-4} M $UO_2(NO_3)_2$. Half an hour later the suspension was centrifuged for 1 hour and uranium was determined in the supernatant fluid. An aliquot of the control sample (3 cc.) with no yeast contained in the supernatant 37 micrograms uranium, while the supernatant of the yeast suspension contained 16 micrograms. A total of 21 micrograms of uranium was taken by the

TABLE VIII

$UO_2(NO_3)_2$ Inhibition of Glucose Oxidation by Bakers' Yeast.
Relation of Yeast Weight to Inhibition

$UO_2(NO_3)_2$, 2×10^{-5} M (14.28 micrograms per vessel). Buffer, veronal, 0.04 M, pH 7.3. Glucose, 0.01 M. Duration of experiments, 40 minutes. Temperature 28°. Figures are blank subtracted (blank, O_2 uptake = 2.2 c.mm. per mg. dry weight per hour).

Yeast Dry weight	O_2 uptake		Inhibition
	Control	$UO_2(NO_3)_2$	
mg.	c.mm.	c.mm.	per cent
7.44	337.5	306.4	9.4
5.58	244.0	211.5	13.3
3.72	164.0	64.0	60.8
1.86	84.3	1.5	98

yeast cells at the saturation level (complete inhibition of glucose oxidation); i.e., 0.2 microgram U per mg. dry weight of yeast cells. Since 1 mg. of yeast cells contained 7.2×10^7 cells, 1 yeast cell would contain at saturation 7.06×10^6 molecules of uranium, not enough to cover the surface of the cell entirely (one yeast cell is from 73 to 388 square micra). One mg. of yeast cells contained 0.376 mg. protein. If the value of 100,000 is taken for the molecular weight of cell membrane protein, one molecule of protein would contain 0.232 molecule of uranium, an indication that only certain protein molecules of the cell membranes combine with uranium. Further evidence of this contention is given by the lack of inhibition of $UO_2(NO_3)_2$ on the activity of yeast saccharase (Fig. 2), an enzyme distributed at the surface of the cell.

Inhibition of glucose oxidation by uranium seemed to be confined to yeast cells. Thus the oxidation of glucose by bacteria such as *Ps. aeruginosa*, *E. coli*, and *M. lysodeikticus* was inhibited only 25 to 19 per cent, while oxidation by *M. creatinovorans* was not affected at all (Table IX).

If inhibition of glucose oxidation was due to combination of uranium with enzymes or coenzymes *in the cell* it would be possible to detect an induction period due to the rate of penetration. Of the numerous experiments devised

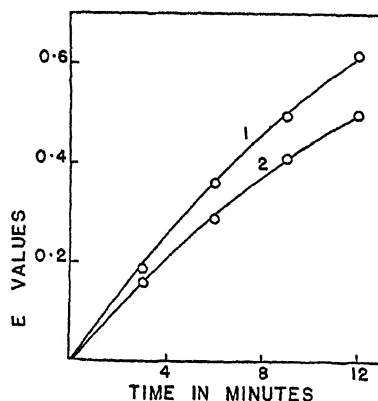


FIG. 2. Effect of $\text{UO}_2(\text{NO}_3)_2$ on the activity of bakers' yeast saccharase. Buffer, hippurate, 0.05 M, pH 3.8. Sucrose, 0.01 M. Temperature 0° . The E values are log $\frac{I_0}{I}$ values for the glucose colorimetric reaction of Nelson (3). (1) Control; (2) $\text{UO}_2(\text{NO}_3)_2$, 1×10^{-3} M.

TABLE IX

Comparative Effect of $\text{UO}_2(\text{NO}_3)_2$ on the Oxidation of Glucose by Yeast Cells and Bacteria

Buffer, veronal pH 7.3, 0.05 M. Glucose, 0.01 M; $\text{UO}_2(\text{NO}_3)_2$, 1×10^{-4} M. Temperature 28° for yeast, 38° for bacteria.

Cells	Weight	O ₂ uptake		Inhibition
		Control	$\text{UO}_2(\text{NO}_3)_2$	
	mg.	c.mm.	c.mm.	per cent
Bakers' yeast	3.0	198	0	Complete
<i>Ps. aeruginosa</i>	4.4	400	300	25
<i>E. coli</i>	4.1	450	338	25
<i>M. lysodeikticus</i>	5.3	94	76.1	19
<i>M. creatinovorans</i>	6.2	53	65	None

to find this induction period all were negative. When glucose and uranyl nitrate were added simultaneously (in experiments with rapid rate of oxidation of glucose), inhibition set in instantaneously. When uranyl nitrate was added 3, 6, and 9 minutes after glucose oxidation had begun, inhibition started also immediately, and the oxygen consumption measured was probably that due

to the glucose which had penetrated into the cells prior to addition of uranium (Fig. 3).

Membrane Permeability and Cellular Oxidations.—Yeast cells are ideal for the study of the role of cell membranes as one of the regulatory mechanisms of cellular metabolism, because they can be kept suspended in buffer solutions varying from pH 2 to pH 8.5 with no change in the rate of their endogenous

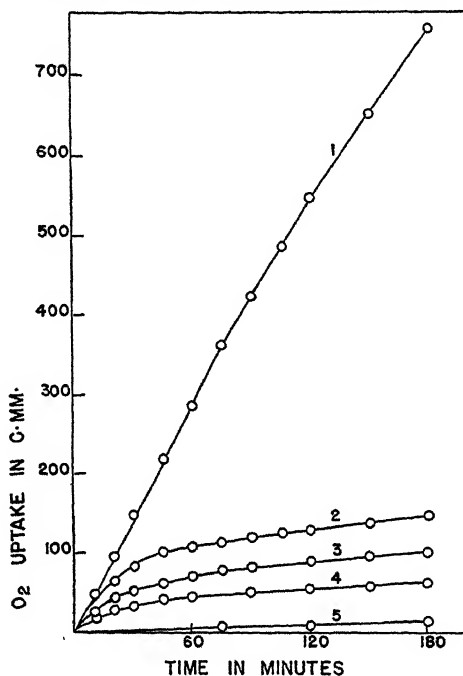


FIG. 3. $\text{UO}_2(\text{NO}_3)_2$ inhibition of glucose oxidation by bakers' yeast cells. Effect of time of addition of $\text{UO}_2(\text{NO}_3)_2$. Buffer, veronal, 0.05 M, pH 7.3. $\text{UO}_2(\text{NO}_3)_2$ 1×10^{-4} M. Glucose, 0.01 M. Temperature 28°. (1) Control; (2) $\text{UO}_2(\text{NO}_3)_2$ added 9 minutes after glucose addition; (3) $\text{UO}_2(\text{NO}_3)_2$ added 6 minutes after glucose addition; (4) $\text{UO}_2(\text{NO}_3)_2$ added 3 minutes after glucose addition; (5) $\text{UO}_2(\text{NO}_3)_2$ and glucose added simultaneously.

respiration. The membrane of the yeast cell was found to be easily permeable to pyruvic acid while the pyruvate ion did not penetrate, as can be seen by the effect of pH on the rate of oxidation of this acid. Pyruvic acid was oxidized at the highest rate at an H^+ ion concentration close to its pK value and oxidation did not become appreciable until 0.4 per cent of it was in the form of the undissociated acid. On the other hand, the behavior of the cell membrane towards acetic acid seemed to vary. While the rate of oxidation of acetic acid was independent of the degree of dissociation of the acid (for the O_2 uptake was

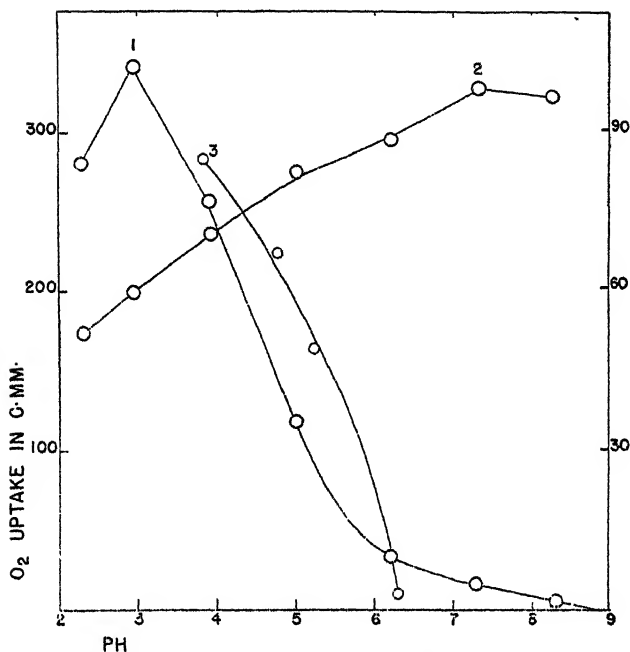


FIG. 4. Effect of pH on the rate of oxidation of pyruvic and acetic acids by bakers' yeast. Buffer, Theorell and Stenhagen's universal buffer; yeast, 10 mg. dry weight. Substrate, 0.01 M. Temperature 28°. Duration of experiment, 1 hour. The figures given are blank subtracted. Blank, 34 to 40 c.mm. O₂ uptake per hour. (1) O₂ uptake in the presence of pyruvic acid. (2) O₂ uptake in the presence of acetic acid. (3) Inhibition of acetate oxidation by fluoroacetic acid (2×10^{-6} M) (per cent inhibition on the right side of ordinate).

TABLE X

Effect of $\text{UO}_2(\text{NO}_3)_2$ on Oxidations Produced by Bakers' Yeast

Buffer, hippurate, 0.025 M, pH 3.8. Substrate concentration 0.01 M. $\text{UO}_2(\text{NO}_3)_2$, 0.00002 M. Duration of experiments, 60 minutes. Temperature 28°. All figures are blank subtracted.

Substrate	O ₂ uptake		Inhibition per cent
	Control	$\text{UO}_2(\text{NO}_3)_2$	
	c.mm.	c.mm.	
Glucose.....	370	53	85.5
Lactate.....	100	70	30
Pyruvate.....	279	233	16
Acetate.....	170	207	None
Citrate.....	23.5	25.9	None
Ethyl alcohol..	399	383	4
Malate.....	13.4	22.1	None

greater when 0.06 per cent of the added acetic acid was as the undissociated acid than when half of it was undissociated), the inhibition of acetate oxidation by fluoroacetic acid was proportional to the degree of undissociation of this acid, an indication that only the undissociated acid penetrates through the cell membrane (Fig. 4).

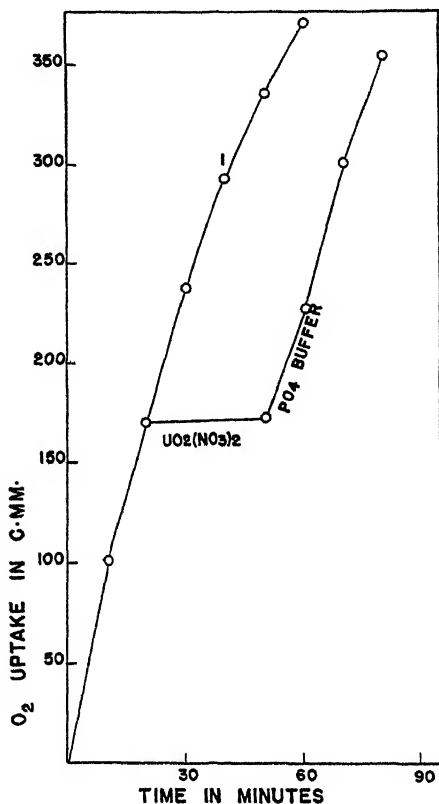


FIG. 5. $\text{UO}_2(\text{NO}_3)_2$ inhibition of the oxidation of lactate by gonococci. Reactivation with phosphate. Buffer, veronal, 0.05 M. Lactate, 0.01 M. $\text{UO}_2(\text{NO}_3)_2$, 0.001 M. Phosphate, 0.01 M added 20 minutes later from the second side arm of the Warburg vessel. Temperature 38°. (1) Control.

Uranium and the Oxidation of Organic Acids.—The oxidation of acetate, citrate, ethyl alcohol, and malate at pH 3.8 was not inhibited at all by a concentration of uranyl nitrate strong enough to inhibit glucose oxidation 85 per cent. The oxidation of lactate was inhibited 30 per cent, and that of pyruvate 16 per cent (Table X).

The inhibition of glucose oxidation by uranium was at first thought to be

due to combination of U with adenosinetriphosphate distributed at the surface of the cell membrane. (The combination of uranyl with adenosinetriphosphoric acid was demonstrated by spectrophotometric measurements.) However, lactate oxidation by yeast, which does not require adenosinetriphosphate, was partially inhibited. The oxidation of lactate by gonococci was completely inhibited by 1×10^{-3} M uranyl nitrate, and the inhibition was completely released on addition of phosphate at a ratio of U:phosphate of 1:10 (Fig. 5). It must be recalled that the oxidation of lactic acid by the enzyme lactic dehydrogenase was not inhibited by U (9).

In cell-free tissue suspensions the oxidation of succinate was inhibited by uranium, an inhibition not affected by the addition of phosphate. The oxida-

TABLE XI

*Effect of $UO_2(NO_3)_2$ on the Oxidation of Succinate by Liver Succinoxidase and by *E. coli**

Buffer, veronal, 0.05 M, pH 7.3. Succinate, 0.04 M. $UO_2(NO_3)_2$, 0.001 M. Temperature 38°. Duration of experiments, 60 minutes for the enzyme and 180 minutes for *E. coli*. Phosphate, 0.01 M, pH 7.3, added 10 minutes after the addition of $UO_2(NO_3)_2$.

System	O ₂ uptake		Inhibition
	Control	$UO_2(NO_3)_2$	
	<i>c.mm.</i>	<i>c.mm.</i>	<i>per cent</i>
<i>Liver Enzyme</i>			
Succinate.....	213	23	89
Succinate + phosphate.....	220	25	89
<i>E. coli</i>			
Succinate (blank subtracted).....	137.6	40.8	70
Succinate + phosphate.....	138	106	23

tion of succinate by *E. coli* was also inhibited by uranium, the inhibition in this case, however, being partially released on addition of phosphate 10 minutes after the addition of uranyl nitrate, an indication that inhibition in the intact cell was not due to combination of the enzyme with U (Table XI).

Uranyl Nitrate and Sea Urchin Sperm.—The inhibitory effect of uranyl nitrate is not confined to the metabolism of yeast cells and bacteria. To study the effect of uranium on isolated animal cells the spermatozoa of sea urchin (*Arbacia punctulata*) were chosen because of their high respiration. The respiration of these cells was inhibited by concentrations of uranyl nitrate, from 1×10^{-3} to 5×10^{-4} M. Half-inhibition was obtained with 1×10^{-4} M (Table XII). Some difficulty was found in the experiments where reversal by citrate and phosphate was studied, because addition of phosphate and citrate to sea water buffered with acetate (artificial sea water containing no calcium) inhibited respiration. To find the extent of release it was therefore necessary to compare

with the respiration of sperm suspended in sea water containing the same amount of citrate or phosphate as that added to the samples containing uranyl nitrate. Under those conditions both citrate and phosphate added at a ratio of 1:10 brought back sperm respiration to that of sperm in citrate or phosphate (Table XIII).

TABLE XII

Effect of $UO_2(NO_3)_2$ on the Respiration of Sea Urchin Sperm

Washed cells suspended in sea water buffered with acetate, 0.05 M. QO_2 values give c.mm. O_2 uptake per mg. dry weight per hour.

$UO_2(NO_3)_2$	pH	Weight of sperm	QO_2		Inhibition
			Control	$UO_2(NO_3)_2$	
M		mg.	c.mm.	c.mm.	per cent
1×10^{-3}	6.44	3.6	10	0.8	92
1×10^{-3}	6.50	2.2	31	3.0	88
1×10^{-3}	6.56	7.9	6.7	1.2	82
5×10^{-4}	6.38	6.6	11.0	1.1	90
5×10^{-4}	6.91	8.3	8.4	0.85	90
5×10^{-4}	6.50	6.25	6.9	0.87	87
1×10^{-4}	6.63	2.3	30.0	14.0	53
5×10^{-5}	6.60	3.7	13.0	10.0	23
1×10^{-5}	6.53	7.0	7.0	6.8	3

TABLE XIII

$UO_2(NO_3)_2$ Inhibition of Sea Urchin Sperm Respiration.

Release with Citrate and with Phosphate

Phosphate (0.05 M), and citrate (0.02 M) added 10 minutes after the addition of $UO_2(NO_3)_2$ 5×10^{-4} M.

Conditions	QO_2		Inhibition
	Control	$UO_2(NO_3)_2$	
	c.mm.	c.mm.	per cent
Sperm cells in acetate.	6.20	0.8	87
Sperm cells in phosphate.	2.50	2.70	None
Sperm cells in acetate.	8.4	0.85	90
Sperm cells in citrate.	5.7	5.80	None

DISCUSSION

The experiments presented in this paper have shown that U in small concentrations can combine reversibly with certain proteins. With plasma albumin the dissociation of the protein-U complex at the alkaline side of the isoelectric point of albumin was readily accomplished on addition of citrate (pH 7.3), while there was no dissociation at pH 3.8. Albumin-U complex was,

however, strong enough not to be dissociated by phosphate at any pH. Similar results were found with enzymes: the inhibition of succinoxidase (from pigeon breast or liver) by uranyl nitrate was released on addition of citrate, while phosphate had no effect at all. When the glucose-fermentation enzymes of brewers' yeast were treated with uranyl nitrate there was inhibition of fermentation, an inhibition which was not abolished on addition of phosphate at a ration of U:P of 1:10.

The experiments with living cells of yeast showed striking differences from those described with uranium and enzymes. The fermentation of glucose by yeast inhibited by uranium was completely released on addition of phosphate or bicarbonate, substances which were unable to release the inhibition of fermentation in cell-free yeast juice. Uranium inhibition of the oxidation of glucose by living yeast was completely released at pH 7.3 on addition of phosphate, citrate, adenosinetriphosphate, and hexosediphosphate. Of these substances, phosphate does not release enzyme inhibitions and the last two do not penetrate across the membrane of yeast cells. If the uranium had penetrated into the cell no release of inhibition could have been produced on addition of adenosinetriphosphate or hexosediphosphate. It must be concluded therefore that inhibition is due not to combination of the metal with enzyme systems within the cell, but rather to adsorption of the metal into the cell membrane making it impermeable to glucose. Inhibition of glucose oxidation by combination with hexokinase or adenosinetriphosphate distributed at the surface of the cell cannot be accepted because (1) hexokinase is not appreciably inhibited by U (9), (2) adenosinetriphosphate addition did not release inhibition at pH 3.8, and (3) such a combination does not explain the inhibition of lactate oxidation.

The inhibition of lactate oxidation by uranium in gonococci, and complete reversal by phosphate, can only be explained as inhibition due to alteration of membrane permeability, because uranium did not inhibit lactate oxidase. The experiments with liver succinoxidase, and oxidation of succinate by *E. coli*, similarly can have no other interpretation. Inhibition of succinoxidase by uranium was not released on addition of phosphate, while phosphate did release partially such inhibition in the intact cell.

These experiments favor the assumption that U combines with the protein portion of the cell membrane and thus renders it less or completely impermeable to the passage of certain oxidizable substrates. This remarkable property of uranium of inhibiting cellular metabolism not by combination with enzymes but by combination with the cell membrane and alteration of its permeability is, we believe, the first example of a new kind of oxidation inhibition, surface inhibitions. Such inhibitions have been postulated by Clark (10), but none of the data presented in favor of this hypothesis were shown to be due to combination of inhibitor with the surface of the cell.

SUMMARY

Uranium as $\text{UO}_2(\text{NO}_3)_2$ combines reversibly with proteins. The degree of dissociation of this combination depends, among other factors, on the H^+ concentration. At pH 7.3 the U-albumin complex was easily dissociated on addition of citrate, while at pH 3.8 it was not. Uranium inhibited reversibly a number of enzyme systems.

Uranium enzyme inhibitions could be reversed on addition of certain hydroxypolycarboxylic acids (citric acid, α -hydroxyaspartic acid, malic acid); in no case, however, did phosphate have any effect.

In cell-free yeast juice, the fermentation of glucose-hexosediphosphate was inhibited by $\text{UO}_2(\text{NO}_3)_2$. Slight reactivation occurred on addition of phosphate.

In living yeast cells, the fermentation and oxidation of glucose was inhibited by small amounts of $\text{UO}_2(\text{NO}_3)_2$ (7.7 micrograms per mg. dry weight), while the oxidation of acetic acid, ethyl alcohol, malic and citric acids, was not affected at all. U inhibition in living yeast cells at pH 7.3 was completely released on addition of small amounts of phosphate, adenosinetriphosphate, and citrate, while at pH 3.8 U inhibition was not released by phosphate and citrate. At saturation, one yeast cell contained 7.06×10^6 molecules of uranium. Lactic dehydrogenase was not inhibited by U while the oxidation of lactic acid by gonococci was inhibited. Addition of phosphate released this inhibition. The U inhibition of liver succinoxidase was unaffected by phosphate, while the U inhibition of the oxidation of succinate by *E. coli* was released by phosphate.

It has been concluded from these experiments that U inhibition of cell metabolism is due to combination of the metal with the protein portion of the cell membrane. Uranium is presented as an example of surface inhibition.

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REGULATORY MECHANISMS OF CELLULAR RESPIRATION

II. THE RÔLE OF SOLUBLE SULFHYDRYL GROUPS AS SHOWN BY THE EFFECT OF SULFHYDRYL REAGENTS ON THE RESPIRATION OF SEA URCHIN SPERM*

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Since the discovery of glutathione by Hopkins (1) a large number of investigators have reported on the importance of sulfhydryl groups in cellular metabolism and in cell division and growth. Of these sulfhydryl groups, the rôle of the —SH groups in certain enzymes, demonstrated for the first time by Hellerman, Perkins, and Clark (2), was extensively investigated by Barron and Singer (3–5) who found that their presence was essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrates, proteins, and fat. The rôle of sulfhydryl groups in cellular division and cell growth, postulated for the first time by Hammett (6), and confirmed by Voegtlin and Chalkley (7), and a number of other investigators (8–11), was demonstrated by quantitative measurements of the sulfhydryl groups by Rapkine (12) and by Chatton Lwoff, and Rapkine (13). More recently Bailey and Perry (14) have reported that the interaction of actin and myosin depends upon the presence of —SH groups in the myosin partner. Whether the —SH groups which are reported to be essential for cellular growth and division are of a protein nature or are peptides like glutathione or free amino acids like cysteine had not yet been determined, although Rapkine has suggested that cell division is preceded by “une dénaturation des protéiques, celle-ci libérant des radicaux sulfhydryles qui en s’oxidant réduiront les groupements —S—S— en groupements —SH.” Oxidation of the —SH groups of enzymes by ionizing radiations (15), and mutations (similar to those found in irradiation) produced by —SH alkylating agents such as sulfur and nitrogen mustards (16, 17) has demonstrated the importance of sulfhydryl groups in biology. We present in this paper experiments on the effect of sulfhydryl reagents on the respiration of sea urchin sperm, which are, in our opinion, proof of the existence in these cells of soluble sulfhydryl groups acting as regulators of cellular metabolism.

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EXPERIMENTAL

Shed sperm of *Arbacia punctulata* was centrifuged for 5 minutes, the supernatant coelomic fluid was pipetted off, and the sperm suspended in sea water in a ratio of one of sperm to 20 of filtered sea water. Of the sulfhydryl reagents used in these experiments, *p*-chloromercuribenzoic acid, 3 times crystallized, was prepared ac-

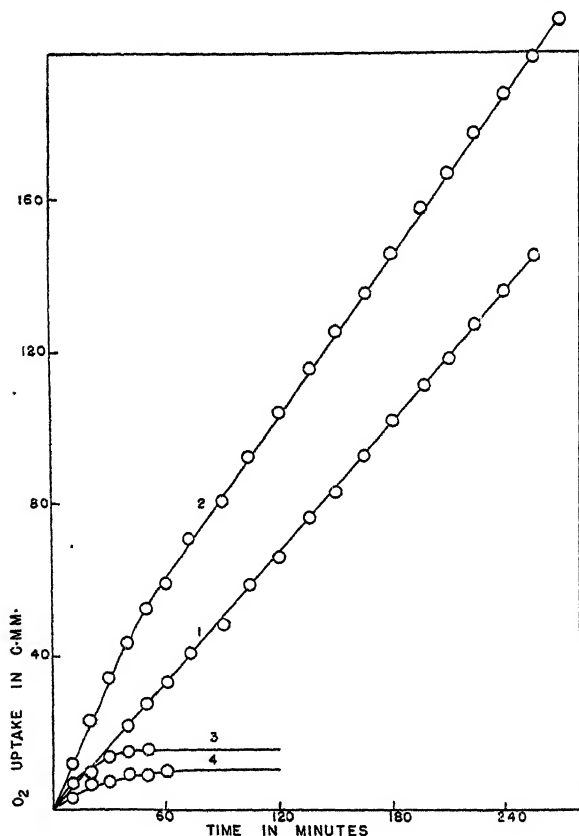


FIG. 1. Effect of iodosobenzoic acid on the respiration of sea urchin sperm. (1) Control; (2) iodosobenzoic acid, 1×10^{-4} M; (3) 3×10^{-4} M; (4) 1×10^{-3} M.

cording to Whitmore and Woodward (18); iodosobenzoic acid, according to Loevenhart and Grove (19); iodoacetamide, according to Anson (20). *p*-Carboxyphenylarsine oxide was kindly provided by Dr. Harry Eagle. The other substances used were good commercial products.

Effect of Sulfhydryl Reagents on the Respiration of Sea Urchin Sperm.—The presence of sulfhydryl groups in the cell can be recognized by the use of oxidizing agents, mercaptide-forming agents, and alkylating agents. None of these

reagents, except some mercaptide-forming agents, is specific for —SH groups. However, if the same results are obtained with the combined use of these three groups of reagents, there is reasonable certainty that the results obtained are due to action on the —SH groups. It is well known that the sperm cells are very rich in —SH groups (21). Furthermore, Barron and Goldinger (22) had shown that iodoacetate increased the respiration of sea urchin sperm. For these reasons, it was decided to use these cells to test the presence of soluble —SH groups acting as regulators of cellular respiration. If such groups did exist together with the fixed —SH groups of the protein moiety of enzymes, there

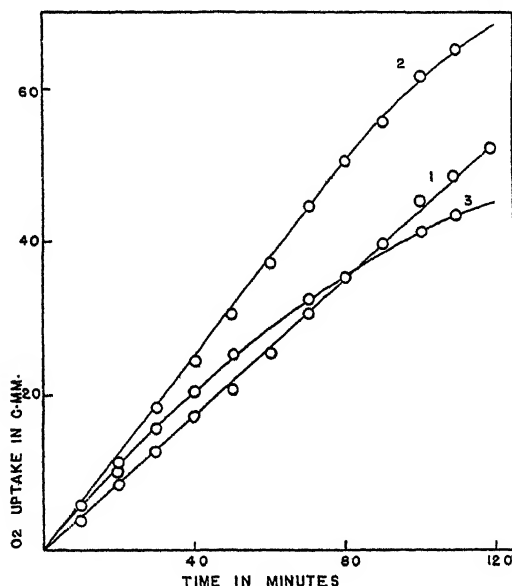


FIG. 2. Effect of iodoacetamide on the respiration of sea urchin sperm. (1) Control; (2) iodoacetamide, 5×10^{-4} M; (3) 1×10^{-3} M.

would occur, on addition of sulfhydryl reagents, at first an increase in the rate of O_2 uptake because of elimination of one of the regulators (the soluble —SH groups), to be followed by an inhibition because of combination with the fixed —SH groups of the sulfhydryl enzymes.

1. *Oxidizing Agents*.—Iodosobenzoic acid was chosen as the oxidizing agent for the —SH groups because Hellerman *et al.* (23) found that this mild oxidizing agent can be used for the titration of cysteine. Iodosobenzoic acid at a concentration of 1×10^{-4} M increased by 70 per cent the respiration of sea urchin sperm. When the concentration was increased to 3×10^{-4} M there was at first a slight rise in respiration, followed by inhibition. When it was increased to 1×10^{-3} M inhibition was the only effect observed (Fig. 1).

2. *Alkylating Reagents*.—Barron and Goldinger (22) found that iodoacetic acid, an alkylating reagent of $-SH$ groups, increased the respiration of sea urchin sperm; and Lardy and Phillips (24) confirmed these findings working with avian sperm. Iodoacetamide, another alkylating reagent, at a concentration of $5 \times 10^{-4} M$ increased the respiration of sea urchin sperm. When the concentration was raised to $1 \times 10^{-3} M$ there was at first some increase in the respiration, which at the end of 80 minutes was followed by inhibition (Fig. 2).

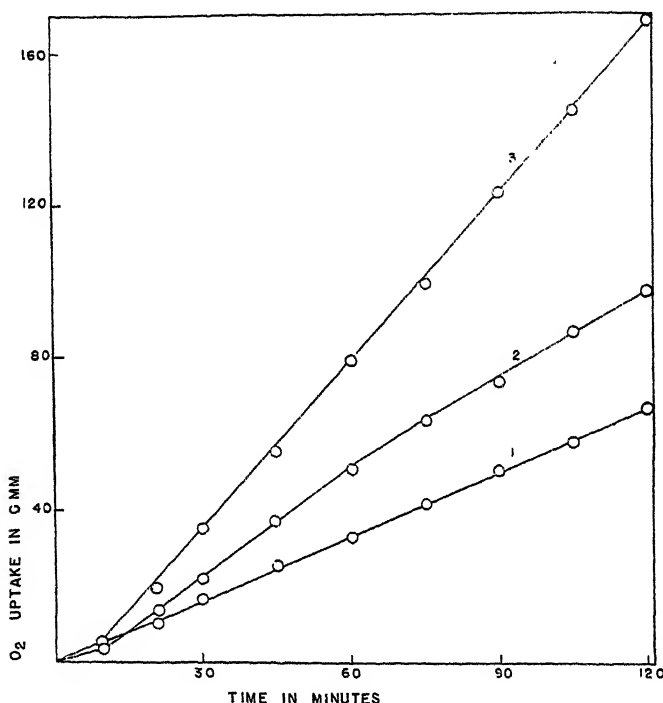
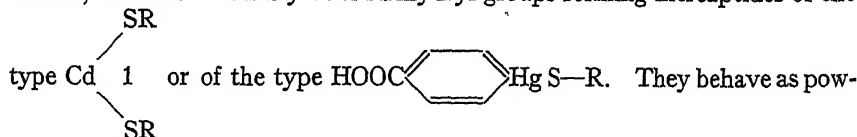


FIG. 3. The effect of $CdCl_2$ on the respiration of sea urchin sperm. (1) Control, (2) $CdCl_2$, $1 \times 10^{-3} M$; (3) $1 \times 10^{-4} M$.

3. *Mercaptide-Forming Reagents*.—A large number of metals, and trivalent arsenic, combine reversibly with sulfhydryl groups forming mercaptides of the



erful inhibitors of $-SH$ enzymes and may be considered as the most specific reagents for sulfhydryl groups. $CdCl_2$ at a concentration of $1 \times 10^{-4} M$ increased 140 per cent the respiration of sea urchin sperm. At the higher concentration of $1 \times 10^{-3} M$, (Fig. 3), $CdCl_2$ increased the respiration only 51 per cent. *p*-Chloro-

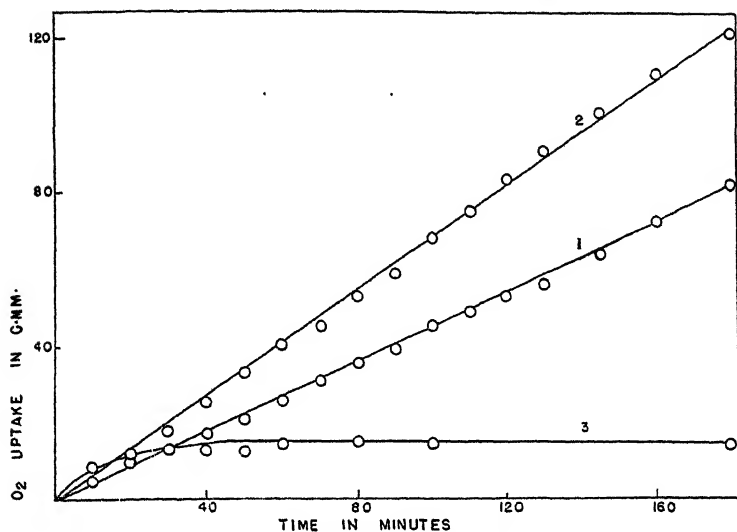


FIG. 4. Effect of *p*-chloromercuribenzoic acid on the respiration of sea urchin sperm. (1) Control; (2) *p*-Cl-Hg-benzoic acid, 1×10^{-4} M; (3) 1×10^{-3} M.

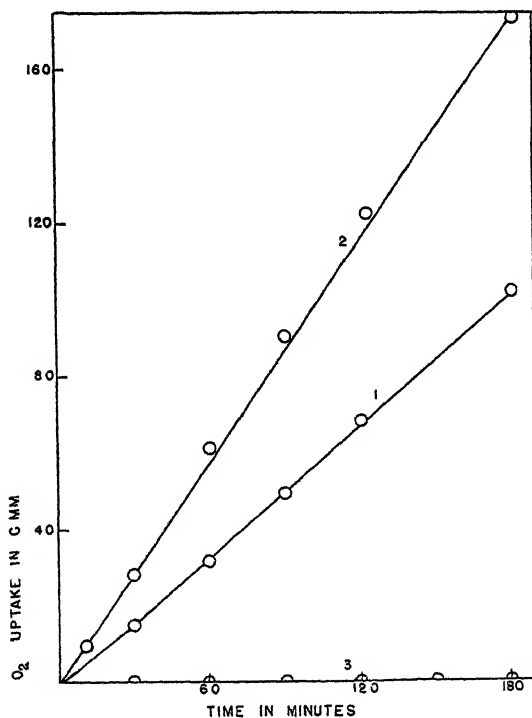


FIG. 5. The effect of HgCl_2 on the respiration of sea urchin sperm. (1) Control; (2) HgCl_2 , 5×10^{-6} M; (3) 1×10^{-4} M.

mercuribenzoate at a concentration of 1×10^{-4} M increased it 57 per cent. When the concentration was increased to 1×10^{-3} M there was at first an increase in the respiration, to be followed by complete inhibition (Fig. 4). HgCl_2 at a concentration of 5×10^{-6} M increased the respiration 88 per cent. When the concentration was raised to 1×10^{-4} M there was complete inhibition (Fig. 5). Sodium arsenite at a concentration of 1×10^{-5} M increased the res-

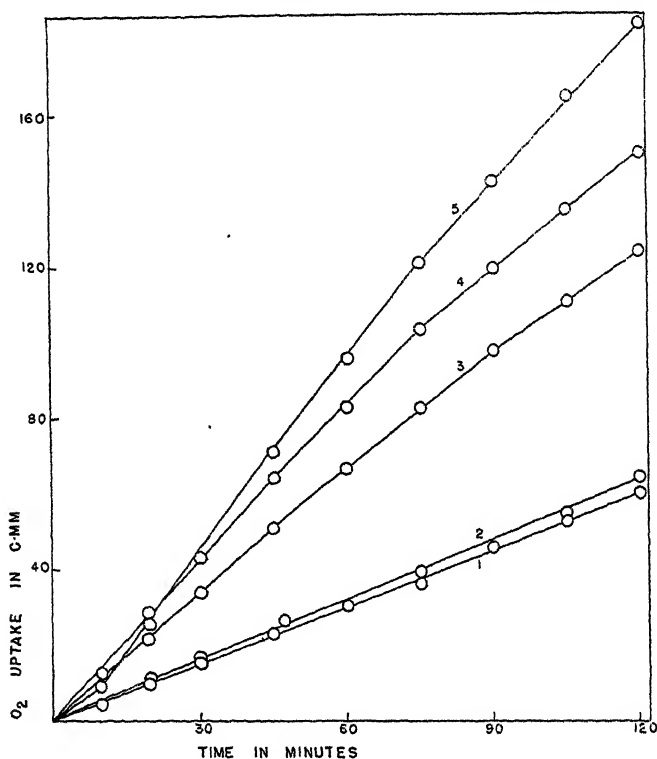


FIG. 6. The effect of Na arsenite on the respiration of sea urchin sperm. (1) Control; (2) Na arsenite, 1×10^{-3} M; (3) 1×10^{-4} M; (4) 2×10^{-5} M; (5) 1×10^{-5} M.

piration 263 per cent. This increase diminished as the concentration increased, so that when the concentration was raised to 1×10^{-3} M there was no effect at all (Fig. 6). The combined process of increase of respiration and inhibition could be studied in one single experiment with *p*-carboxyphenylarsine oxide, which seems to penetrate the cell membrane quite slowly. At a concentration of 3×10^{-3} M this arsenical produced a definite increase in respiration, which at the end of 30 minutes reached 100 per cent. From this time, O_2 uptake decreased exponentially so that at the end of 120 minutes there was almost complete inhibition (Fig. 7).

A comparison of the effect of these three groups of sulfhydryl reagents was made with iodoacetamide, *p*-chloromercuribenzoate, and iodosobenzoate, all at a concentration of 5×10^{-4} M. Iodoacetamide maintained the increase in the respiration for the duration of the experiments (100 minutes); *p*-chloromercuribenzoate increased the respiration definitely for the first 30 minutes and produced complete inhibition 40 minutes later; iodosobenzoate produced only a slight increase in respiration, which at the end of 30 minutes was replaced by

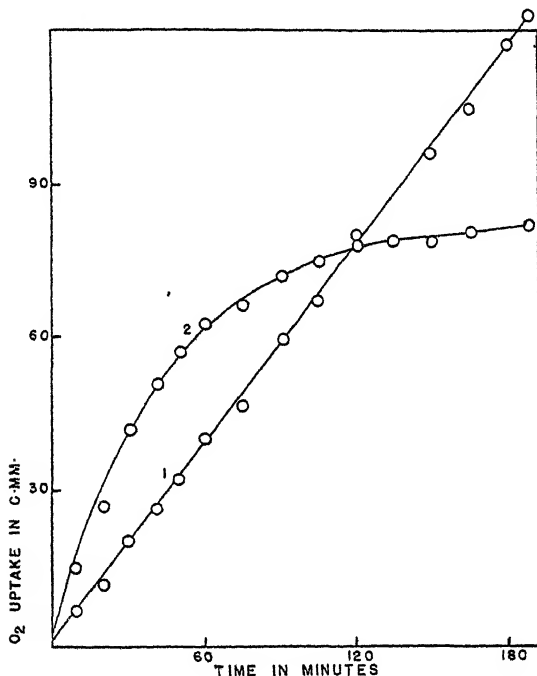


FIG. 7. Effect of *p*-carboxyphenarsine oxide (3×10^{-3} M) on the respiration of sea urchin sperm. (1) Control; (2) *p*-carboxyphenarsine.

complete inhibition (Fig. 8). At equal concentrations the series of events (increase in respiration and inhibition), as well as the duration of the increased respiration, must be determined by the rate of penetration of the sulfhydryl reagents.

An attempt was made to reverse the increase in respiration produced by mercaptide-forming agents through the addition of sulfhydryl compounds which may combine with the reagents, thus liberating the cellular sulfhydryl groups. Since the inhibition of succinoxidase by CdCl_2 is completely reversed on addition of 2,3-dimercaptopropanol (BAL) (25), the respiration of sea urchin sperm was measured in the presence of 1×10^{-4} M CdCl_2 , 1×10^{-3} M

BAL, and CdCl_2 and BAL added 15 minutes later. The increase in respiration produced by CdCl_2 (55 per cent) was reduced to 17 per cent on addition of

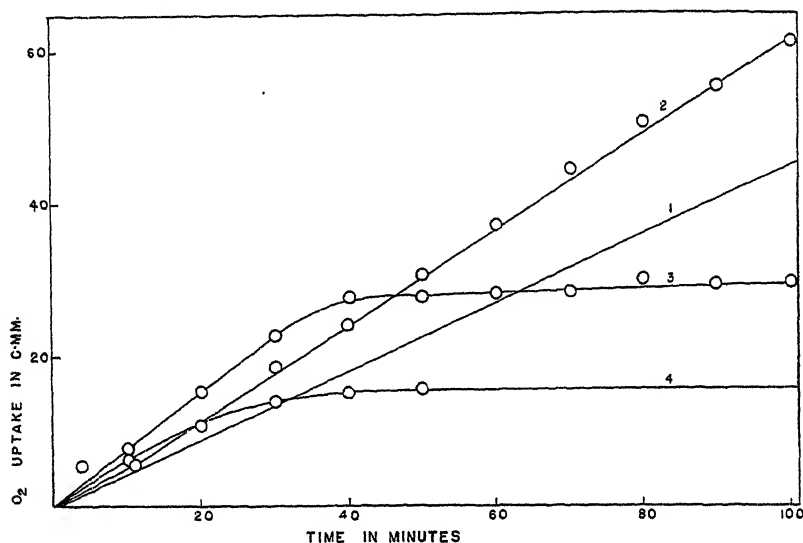


FIG. 8. Effect of sulfhydryl inhibitors on the respiration of sea urchin sperm. —SH inhibitor concentration, 5×10^{-4} M. (1) Control; (2) iodoacetamide; (3) *p*-chloromercuribenzoate; (4) iodosobenzoate.

TABLE I

The Reversal of CdCl_2 Increase in Respiration by BAL

Sea urchin sperm suspended in sea water. CdCl_2 , 1×10^{-4} M. 2,3-dimercaptopropanol (BAL), 1×10^{-3} M. BAL was added 15 minutes after the addition of CdCl_2 . Temperature 25° . Duration of experiments, 90 minutes.

Additions	O ₂ uptake	Increase
	c.mm.	per cent
None.....	45	—
CdCl_2	70	55
BAL.....	60	33
CdCl_2 + BAL ...	54	17

BAL (Table I). If the O_2 uptake of BAL is subtracted, the reversal may be considered complete.

Effect of Other Inhibitors of Cell Respiration.—Other inhibitors of cellular respiration which do not combine with sulfhydryl groups were also tested. HCN was found to inhibit completely sea urchin sperm respiration at a concentration of 1×10^{-4} M. Even a concentration of 1×10^{-7} M inhibited the

TABLE II

Effect of HCN on the Respiration of Sea Urchin Sperm

Sperm suspensions in sea water; temperature 25°. Duration of experiments, 60 minutes

HCN	O ₂ uptake	Inhibition
M	c.mm.	per cent
None	35.3	—
1×10^{-4}	1	97
1×10^{-5}	4.8	86.4
5×10^{-6}	13.5	61.7
1×10^{-7}	20.4	42
1×10^{-8}	35.0	None

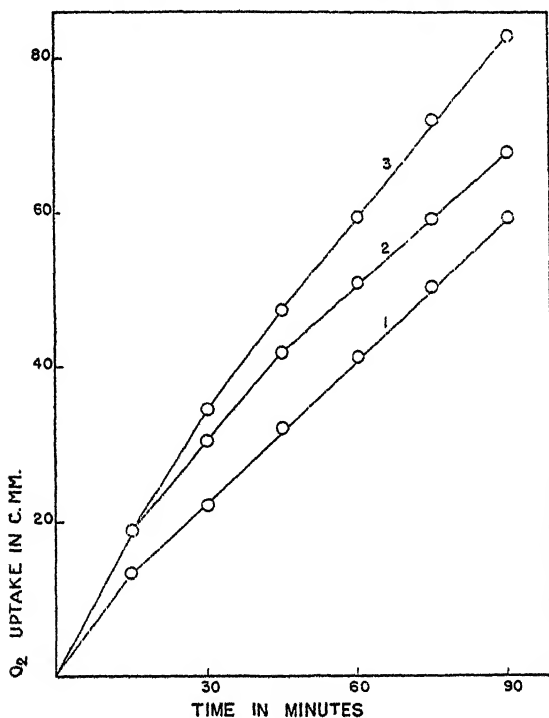


FIG. 9. The effect of Na azide on the respiration of sea urchin sperm. Sperm suspended in acetate buffer pH 6.8 (0.03 M). (1) Control; (2) Na azide, 1×10^{-3} M; (3) Na azide, 5×10^{-3} M.

respiration 42 per cent. When the concentration was diminished to 1×10^{-8} M HCN had no effect at all (Table II). In no case was there an increase in respiration. With sodium azide, under similar conditions (*i.e.* with the sperm cells suspended in sea water) no effect on the respiration of sea urchin sperm

was observed with concentrations varying from 0.01 M to 0.001 M. When the sperm cells were suspended in sea water containing acetate at a pH value of 6.8, sodium azide (5×10^{-3} M) increased the respiration by 38 per cent. When the concentration was diminished to 1×10^{-3} M the increase diminished also (Fig. 9). Sperm cells utilize acetate, as shown by the increase in O_2 uptake on addition of acetate (42 per cent). Perhaps sodium azide orients the acetate metabolism of sperm towards a more complete oxidation, as it does in yeast (26).

Urethanes were shown by Meyerhof (27) to inhibit the respiration of sea urchin eggs. A saturated solution of phenylurethane inhibited 60 per cent the respiration of sea urchin sperm; at half-saturation, the inhibition decreased to

TABLE III

Effect of Urethanes on the Respiration of Sea Urchin Sperm

Sperm suspended in sea water. Temperature 25°. Duration of experiments, 90 minutes.

Urethane	Concentration	O_2 uptake	Increase (+) or inhibition (—)
	M	mm.	per cent
None.....	—	43	—
Phenylurethane.....	Saturated solution	17	—60
Phenylurethane.....	$\frac{1}{2}$ saturation solution	26	—39
Phenylurethane.....	$\frac{1}{4}$ saturation solution	20.8	—12
Phenylurethane.....	$\frac{1}{10}$ saturation solution	43	None
None.....	—	62.4	—
Ethylurethane.....	0.1	76.8	+23
Ethylurethane.....	0.01	63	None
Ethylurethane.....	0.001	64	None

39 per cent; at one-fourth saturation the inhibition was only 12 per cent; at one-tenth saturation it had no effect at all. Ethylurethane at a concentration of 0.1 M increased by 23 per cent the respiration of sea urchin sperm; when the concentration was diminished to 0.01 M this small increase disappeared (Table III).

DISCUSSION

The experiments presented in this paper on the increase in the respiration of sea urchin sperm produced by small concentrations of sulfhydryl reagents, and inhibition of respiration when the concentrations are increased can be satisfactorily explained by assuming that there are in the cell two types of sulfhydryl groups: *soluble* sulfhydryl groups (glutathione and substances similar to it) distributed throughout the cell, and *fixed* sulfhydryl groups; *i.e.*, sulfhydryl groups in the side chains of the protein moiety of enzymes which are essential

for the activity of certain enzymes. The soluble sulfhydryl groups, being dissolved in the water phase of the cell, would contribute in a great measure to the maintenance of the oxidation-reduction equilibrium of the cell. By virtue of their very negative oxidation-reduction potential (E'_0 of cysteine at pH 7 = -0.39 v. according to Borsook *et al.* (28)) and their reversibility in the cell (29), the soluble sulfhydryl groups must regulate the rate of respiration by inhibiting the rate of reoxidation of the cytochrome system in the same manner as dimercaptopropanol (BAL) inhibits the rate of oxidation of reduced cytochrome C by cytochrome oxidase (30). Addition to the cells of sulfhydryl reagents in small concentrations will produce an increase in cellular respiration because of combination with these soluble sulfhydryl groups which readily react with them and thus produce an abolition of this regulating mechanism of cellular respiration. As the concentration of sulfhydryl reagents is increased they will combine with the fixed sulfhydryl groups belonging to the side chains of proteins and many of them essential for enzymatic activity. Inhibition of respiration will be the consequence. By this diminution in the rate of cellular oxidations, the soluble sulfhydryl groups contribute to the orientation of cellular metabolism towards synthesis towards anabolic processes. The reversible inhibition of cell division by HgCl_2 (12) and by Cu^{++} (31) is evidence of the rôle of sulfhydryl groups in developmental growth. Further evidence for the influence of these compounds may be found in the increased concentration of sulfhydryl groups in fast growing cells and in cells during the process of division. We believe these sulfhydryl groups belong mainly to the type of *soluble* —SH groups and not to the type of *fixed* —SH groups of proteins, as was postulated by Rapkine (12). The rapid combination with the sulfhydryl reagents, as shown in these experiments long before there is combination with the —SH groups of enzymes, speaks in favor of this assumption.

SUMMARY

Oxidizing agents of sulfhydryl groups such as iodosobenzoate, alkylating agents such as iodoacetamide, and mercaptide-forming agents such as cadmium chloride, mercuric chloride, *p*-chloromercuribenzoate, sodium arsenite, and *p*-carboxyphenylarsine oxide, added in small concentrations to a suspension of sea urchin sperm produced an increase in respiration. When the concentration was increased there was an inhibition. These effects are explained by postulating the presence in the cells of two kinds of sulfhydryl groups: *soluble sulfhydryl groups*, which regulate cellular respiration, and *fixed sulfhydryl groups*, present in the protein moiety of enzymes. Small concentrations of sulfhydryl reagents combine only with the first, *thus* producing an increase in respiration; when the concentration is increased, the fixed sulfhydryl groups are also attacked and inhibition of respiration is the consequence.

Other inhibitors of cell respiration, such as cyanide and urethanes, which

do not combine with —SH groups, did not stimulate respiration in small concentration.

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THE CHEMICAL STATE OF THE CALCIUM REACTING IN THE COAGULATION OF BLOOD*

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For nearly a half century almost every student of coagulation has accepted the hypothesis that ionized calcium is essential for coagulation. Many investigators, moreover, hold that ionized calcium acts as a catalyst. The origin of these concepts can be traced first to Arthus and Pagès (1) who in 1890 showed that blood was made incoagulable by sodium oxalate and that readdition of calcium restored normal coagulation. They justifiably concluded that calcium was necessary for the coagulation reaction. Two years later Pekelharing (11) found that sodium citrate was equally effective as an anticoagulant, and, being no doubt influenced by the work of Arthus and Pagès, he concluded that the action of sodium citrate was on the calcium. Interestingly, Alexander Schmidt (18) refused to accept the idea that calcium had a specific rôle in coagulation. He attributed the action of both sodium oxalate and sodium citrate to a non-specific salt effect.

Considerable uncertainty remained until Sabbatani (17) explained the peculiar behavior of sodium citrate by stating that calcium forms a complex with this compound whereby its ionization is depressed, and he postulated that ionized calcium is essential for coagulation.

Since Hammarsten (4) had previously found that calcium was necessary for the conversion of prothrombin to thrombin, it became generally accepted that ionized calcium was required for the activation of prothrombin. Morawitz (8) and Fuld and Spiro (3) incorporated the concept that ionized calcium acted catalytically in their "classical theory" of coagulation. Thereafter few investigators had the temerity or sufficiently unconventional minds to question the indispensability of ionized calcium.

It is well established that part of the plasma calcium is free or ionized and part is bound and non-diffusible. It has been estimated by various methods that normally about one-half of the total calcium is bound or unionized. When Vines (24) found that only one-seventeenth of the total calcium present in serum was required to bring about a normal coagulation time of decalcified

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blood, he concluded that only bound calcium participated in coagulation. Scott and Chamberlain (19) reported similar findings. More recently Nordbø (9) found that 0.15 to 0.18 mM of calcium per liter of plasma was necessary to cause coagulation. This amount of calcium is only one-fifteenth of the total found in plasma; nevertheless, he assumes that this fraction is ionized, because it was ionized calcium which he added to decalcified plasma. Ransmeier and McLean (16) determined that the minimal Ca^{++} concentration required for the coagulation of citrated plasma was 0.28 mM per liter of human plasma and 0.18 mM per liter of dog plasma. These values are similar to those of Nordbø who worked with beef and horse plasma. In both studies it was assumed that it was the minimum amount of calcium required to initiate coagulation that was significant.

The introduction by Steinberg (22) of a resin, amberlite, which quantitatively removes calcium from the blood, has made possible a new approach to the problem of calcium in coagulation. By employing this new reagent, Quick (14) found that the minimum amount of calcium required to produce a normal prothrombin time, which is the real criterion of maximum and optimal coagulation, is 0.0012 M for human and 0.0004 M for dog plasma. It was found (15) further that if the amount of calcium added is below these levels, the amount of prothrombin consumed or converted is quantitatively reduced even though thromboplastin is in excess. Neither of these findings are in accord with the theory that calcium acts ionically and plays the rôle of a catalyst.

From this short historical review it should be obvious that the concept that it is ionized calcium in the rôle of a catalyst which is responsible for the activation of prothrombin, is still a theory and not an established fact. Its main support is the assumption that sodium citrate acts as an anticoagulant by depressing the ionization of calcium. It is the purpose of this paper to reexamine critically the rôle of calcium in the coagulation mechanism by three different approaches: (1) the decalcifying action of sodium oxalate; (2) the anticoagulant action of sodium citrate; and (3) the influence of calcium on the stability of the labile factor of prothrombin.

EXPERIMENTAL

Determination of Prothrombin Time

The method of Quick was used. The thromboplastin prepared by dehydration of rabbit brain with acetone consistently yielded a prothrombin time of $11\frac{1}{2}$ to 12 seconds for human blood.

Preparation of Stable Native Plasma

Blood was collected by venipuncture using a needle and syringe coated with silicone (General Electric dri film 9987). The syringe was kept in ice prior to use, and the blood was immediately transferred to silicone-coated tubes immersed in an ice bath,

and covered with mineral oil. The chilled blood was centrifuged for 15 minutes at 4,000 R.P.M. in an angle centrifuge at refrigerator temperature. The plasma was removed with a silicone-coated pipette and transferred to a silicone-coated test tube.

Preparation of Tricalcium Phosphate

To a solution containing 158 gm. of trisodium phosphate in 1 liter of distilled water, an equal volume of calcium chloride solution containing 66.6 gm. of the anhydrous salt was added slowly with vigorous stirring. The pH was adjusted to 7. The precipitate, $\text{Ca}_3(\text{PO}_4)_2$, was washed by decantation until the sodium chloride was removed. The suspension was made up to 1 liter and it therefore had a concentration of 0.2 M. From this stock suspension, a 0.008 M preparation was made by diluting 4 cc. with 96 cc. of distilled water. One cc. of the dilute suspension was transferred to a small test tube which was centrifuged to pack the tricalcium phosphate. The water was poured off, the tube drained, and 1 cc. of plasma added. The tricalcium phosphate and plasma were mixed and allowed to stand at room temperature for 10 minutes. The adsorbent was removed by centrifugation.

The Removal of Calcium with Amberlite

The method as outlined by Quick (14) and Stefanini (21) was used.

Quantitative Measurement of the Action of Sodium Oxalate on Prothrombin Activity

Stewart and Percival (23) and later Nordbö obtained evidence that the sodium oxalate did not inhibit coagulation instantaneously, but required considerable time. In 1940 Quick (12) studied this action quantitatively by measuring the increase of the prothrombin time of plasma after the addition of an accurately measured amount of sodium oxalate. He found that the speed of inhibition increased in proportion to the amount of oxalate added. Because of the importance of these results, the experiment was repeated with greater precision and on a larger number of plasmas. The data are presented in Table I.

It will be observed that the addition of 0.02 cc. of 0.1 M sodium oxalate to 1 cc. of human plasma caused only a slow increase in the prothrombin time. Yet, this amount of sodium oxalate brings the concentration to 0.002 M in the plasma. Since the calcium level averages 10 mg. per 100 cc. which is 0.0025 M, the amount of sodium oxalate added is sufficient to precipitate approximately 80 per cent of the total calcium. Since only half of the calcium is ionized and since this fraction is rapidly removed as insoluble calcium oxalate, it appears unlikely that the slow increase in prothrombin time is due to the removal of ionized calcium. Even when the sodium oxalate is increased to 0.004 M (by adding 0.04 cc. to 1 cc. of plasma) which is 60 per cent more than is required to precipitate the total calcium, the diminution of prothrombin activity is slow and one-half hour after the oxalate is added the plasma still clots in 330

seconds. Under these conditions, the ionized calcium can come only from dissolved calcium oxalate. Since the solubility is less than 0.0006 gm. per 100 cc., the concentration of ionized calcium is below 0.000004 M, which is far less than the values calculated as minimal by Nordbö and by Ransmeier and McLean.

The most logical explanation for the action of sodium oxalate is that instead of only precipitating ionized calcium, it also removes calcium from a compound which is essential for coagulation. Apparently this compound has a great avidity for calcium and therefore a relatively large excess of sodium oxalate is required to decalcify it completely. An examination of the data of Table I

TABLE I

The Speed of Inhibition of the Prothrombin Activity by Sodium Oxalate and Sodium Citrate

Time after mixing....	Prothrombin time					
	0	10 sec.	1 min.	5 min.	10 min.	30 min.
	sec.	sec.	sec.	sec.	sec.	sec.
Amount of reagent added to 1 cc. of plasma						
Sodium oxalate 0.1 M						
0.02 cc.	12	12	12½	14	18	*
0.04 cc.	12	12	18	52	165	330
0.06 cc.	12	17	75	∞		
Sodium citrate 0.1 M						
0.02 cc.	12	12½	12½	*		
0.04 cc.	12	15	15	14	13	*
0.06 cc.	12	28	28	27½	25	*
0.08 cc.	12	135	150	135	135	125

* Spontaneous coagulation.

clearly indicates that the reaction follows the law of mass action. Since this compound has a great affinity for calcium, it is logical to assume that when ionized calcium is added to oxalated or otherwise decalcified plasma, a prompt resynthesis of this calcium compound occurs with an immediate restoration of the coagulability of the plasma especially if an excess of thromboplastin is present. This temporary coagulability in the presence of sodium oxalate has been interpreted by both Nordbö (9) and Owren (10) as due to the added ionized calcium remaining as such for a short period. Quick (14), however, showed that this activity remains considerably longer than the time required for the complete precipitation of ionized calcium as the oxalate salt.

It is difficult to find a simpler and more obvious explanation for the slow inhibitory action of sodium oxalate than the one offered; namely, that it re-

moves calcium from a compound which is essential in the conversion of prothrombin to thrombin, but which is inactive in its decalcified state. Sodium citrate appears to act in an entirely different manner. It exerts its maximum depressing action quickly and it only gradually becomes more inhibitory as its concentration is increased. The nature of the action of sodium citrate is shown in the following experiments.

The Action of Sodium Citrate on Prothrombin

Bordet and Delange (2) discovered that tricalcium phosphate removes by adsorption a plasma constituent essential for coagulation. They concluded that it was serozyme (prothrombin). Subsequently other agents such as barium sulfate, magnesium hydroxide, and aluminum hydroxide were found which likewise remove prothrombin by adsorption. It was generally accepted

TABLE II

The Blocking Action of Sodium Citrate on the Absorption of Prothrombin by Tricalcium Phosphate

Molarity of sodium citrate of which 1 volume was added to 9 volumes of plasma....	Prothrombin time after absorption with $\text{Ca}_3(\text{PO}_4)_2$										
	Control*	0.25	0.20	0.15	0.12	0.10	0.08†	0.06†	0.04†	0.02†	0
	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.	sec.
Rabbit.....	7	7	7½	10	11½	13½	17	18	21	25	∞
Dog.....	5½	5½	5½	5½	5½	6	8	10	18	48	∞
Man.....	12	12	12	12	12	13½	15½	19	33	135	∞

* Control. Prothrombin time of oxalated plasma not treated with $\text{Ca}_3(\text{PO}_4)_2$.

† For low concentrations of sodium citrate, oxalated plasma in place of native plasma was used to prevent spontaneous coagulation.

that the prothrombin thus removed was a unitary substance which required only thromboplasin and calcium for its activation. This concept lost its validity when in 1943 Quick (13) found that prothrombin, or perhaps more accurately, the chemical aggregate on which prothrombin activity depends, consists of a complex, only part of which is adsorbable by aluminum hydroxide.

To avoid confusion, however, the term prothrombin, will be reserved in this paper for the adsorbable fraction while the designation of the labile factor will be applied to the non-adsorbable part of the complex which diminishes when plasma is stored.

The adsorption of prothrombin by tricalcium phosphate which occurs readily from oxalated plasma fails to take place when sodium citrate is used. There is moreover a definite quantitative relationship between the concentration of sodium citrate and the non-adsorbability of prothrombin. This relationship was studied and the results obtained are recorded in Table II. It can be seen that enough sodium citrate must be added to human blood to produce a final

concentration of a little more than 0.01 M before all the prothrombin remains non-adsorbable. Below that concentration some of the prothrombin is removed by tricalcium phosphate and therefore the prothrombin time becomes prolonged. Rabbit blood requires a somewhat higher concentration of sodium citrate, approximately 0.02 M to prevent completely adsorption of prothrombin, whereas dog blood is similar to human blood in its response to sodium citrate.

This finding clearly suggests that sodium citrate combines with prothrombin and thereby blocks its adsorption with agents such as tricalcium phosphate. Such a union obviously does not occur between prothrombin and the oxalate radical. To rule out the possibility that prothrombin itself is not adsorbable but may become so after decalcification by sodium oxalate, several studies (recorded in Table III) were made. Thus, native hemophilic plasma when treated with tricalcium phosphate loses all prothrombin activity, but if mixed

TABLE III
Prothrombin Time of Plasma (in Seconds)

	Native	Amberlite-treated	Oxalated
Control*.....	12	12	12
After treatment with $\text{Ca}_3(\text{PO}_4)_2$	∞	∞	∞
After addition of sodium citrate and treatment with $\text{Ca}_3(\text{PO}_4)_2$ †.....	12	12	12

* Control. 0.1 cc. of plasma, 0.1 cc. of thromboplastin, and 0.1 cc. of 0.02 M CaCl_2 .

† To 0.9 cc. of plasma, 0.1 cc. of 0.2 M sodium citrate was added.

with sodium citrate before the addition of the adsorbing agent, it retains its prothrombin completely. Blood treated with amberlite, the agent that removes calcium and so renders it incoagulable, will when treated with tricalcium phosphate lose its prothrombin activity. If however, sodium citrate is added, the adsorbent no longer is able to take out the prothrombin. In fact the adsorption is blocked even in oxalated plasma if sodium citrate in sufficient amounts is added (Tables II and III). The removal of prothrombin by tricalcium phosphate is therefore independent of calcium.

The conclusion that prothrombin forms a union with sodium citrate and thereby loses its adsorbability can be accepted without hesitation, but the moot question remaining is: does this combination with citrate cause the prothrombin to become inactive? In other words, is sodium citrate an antiprothrombin? The difficulty in arriving at a solution of this problem is that calcium also forms a complex with the citrate radical, as a result of which calcium ionization is depressed. If one considers the low Ca^{++} in the oxalated plasma of Table I which still permits coagulation, it seems unlikely that the concentration of

citrate required to inhibit coagulation is sufficient to depress Ca^{++} below this effective level since the primary dissociation constant of calcium citrate is fairly high. According to Hastings, McLean, and their associates (5) the $\text{pK}_{\text{Ca citrate}}$ is 3.22. Further work will, however, be required before a final answer can be given.

It follows that one must consider how the addition of calcium chloride to citrated plasma restores coagulation. According to the old and generally accepted concept, it merely furnished calcium ions in excess to the ones depressed by the citrate. On the basis of evidence that prothrombin combines with the

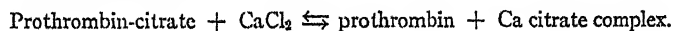
TABLE IV

The Prothrombin Time Obtained on Citrated Plasma by Substituting Magnesium and Strontium Chloride for Calcium Chloride*

Molarity of sodium citrate (1 volume added to 9 volumes of blood).....		Prothrombin time				
		0.2	0.15	0.1	0.075	0.05
		sec.	sec.	sec.	sec.	sec.
CaCl_2	0.01 M	45	15	11	11	11
	0.02 M	11	11	12	12	12
MgCl_2	0.01 M	—	180	30	14	14
	0.02 M	120	32	15	14	13½
	0.03 M	36	22	16	16	17
SrCl_2	0.01 M	—	60	25	16	14
	0.02 M	58	25	16	16	14½
	0.03 M	26	22	17	18	16

* The citrated blood was kept in ice bath until tested.

citrate radical, a new explanation can be offered; namely, calcium chloride removes the citrate from prothrombin thereby restoring it to its original state. This can be expressed as follows:



To be sure, the Ca^{++} also are increased.

If this concept is correct, magnesium and strontium should also restore coagulation when added to citrated plasma. The results given in Table IV show that both possess the power to do this. Strontium itself has a much weaker coagulation action than calcium while magnesium has only a feeble activity as Stefanini and Quick (20) have recently shown. Their action can therefore be explained either as liberating prothrombin from the citrate radical or increasing the Ca^{++} by coming in equilibrium with calcium citrate. The equations are:

(1) Prothrombin-citrate + $\text{MgCl}_2 \rightleftharpoons$ prothrombin + Mg citrate complex.

(2) Ca citrate + $\text{MgCl}_2 \rightleftharpoons$ Mg citrate + CaCl_2
 \downarrow
 Ca^{++} .

The fact that strontium and magnesium become less effective as the citrate concentration is increased is in the favor of the first explanation. The greater the concentration of citrate the firmer its union with prothrombin and the less complete the liberation of prothrombin. Were the action of magnesium chloride merely one of increasing Ca^{++} , slight changes in the citrate concentration should be without any appreciable effect on the coagulation time.

Thus the restoration of coagulation in citrated plasma by the addition of calcium chloride can be explained as satisfactorily by the removal of citrate from prothrombin as by increasing the Ca^{++} . Nevertheless, a definitive solution will require additional data obtained by more direct experimental approaches.

The Relation of the Stability of the Labile Factor to the Calcium Concentration of the Plasma

When oxalated human plasma is stored in an open container at ordinary refrigerator temperature, prothrombin activity diminishes relatively rapidly whereas undecalcified or native plasma (such as hemophilic which remains fluid) fails to show this loss (13, 20). Likewise, plasma from blood mixed with 0.1 M sodium citrate (1 volume to 9 volumes of blood) does not show this rapid and marked increase in prothrombin time when stored, but if the concentration of sodium citrate is increased to 0.2 M the disappearance of prothrombin activity is similar to that occurring in oxalated plasma.

In Table V the disappearance of prothrombin activity from plasma under various types of conditions is studied. It will be observed that the prothrombin time increases in 24 hours from a normal of $11\frac{1}{2}$ to 12 to 14 to 15 seconds in oxalated, 0.2 M citrated and amberlite-treated plasma, whereas a decrease or an insignificant increase occurs in the 0.1 M citrated and native plasma. In two plasmas (oxalated and amberlite-treated) in which diminution of prothrombin activity occurs, one is certain that decalcification has taken place. In the native plasma in which the calcium is unchanged, the prothrombin remains essentially unaltered. This clearly suggests that the labile factor, which is responsible for the decrease of prothrombin activity, is stable in the presence of calcium, and is inactivated or destroyed in decalcified plasma. Whether the labile factor itself is a calcium compound cannot be decided until more experimental information is available.

The important suggestion accruing from the observation of the difference in effect of 0.1 M and 0.2 M sodium citrate is that the latter depresses the calcium

ion concentration sufficiently so that the labile factor diminishes, while the weaker concentration of sodium citrate does not. When 0.1 sodium citrate is added to amberlite-treated plasma which is already deprived of calcium, the rate of prothrombin diminution is little affected. This clearly indicates that the stability of the labile factor in 0.1 M sodium citrate plasma is due to insufficient decalcification. In other words, sufficient calcium remains to protect the labile factor. Since even a lower concentration than 0.1 M is enough to inhibit the coagulation of human plasma, an appreciable Ca^{++} concentration must still be present at the citrate level at which incoagulability occurs.

TABLE V

The Influence of the Anticoagulant on the Loss of Prothrombin Activity during Storage

Anticoagulant	Volume used	Volume of blood	Prothrombin time		
			Immediate	After 24 hrs.	After 48 hrs.
	cc.	cc.	sec.	sec.	sec.
Sodium oxalate 0.1 M.....	0.3	2.7	12	15	17½
Sodium citrate 0.1 M.....	0.3	2.7	11½	10	11
Sodium citrate 0.2 M.....	0.3	2.7	12	14	15
Amberlite IR-100.....	—	3.0	11½	14	15
Amberlite IR-100 + sodium oxalate 0.1 M*.....	0.3	2.7	11½	14	18
Amberlite IR-100 + sodium citrate 0.1 M*.....	0.3	2.7	11½	14½	18
None†.....	—	3.0	12	12½	13

* The sodium oxalate or citrate was added to blood which was previously treated with amberlite IR-100.

† By collecting blood in silicone-coated glassware and centrifuging it at high speed, plasma was obtained which remained fluid in silicone-coated test tubes.

Two important facts: (1) the indispensability of the labile factor in coagulation and (2) its instability in decalcified plasma should ultimately serve as important keys to the rôle of calcium in the formation of thrombin.

DISCUSSION

The almost universally accepted idea that it is the ionized calcium of the blood that participates in the coagulation reaction and that it functions as a catalyst is a theory and by no means an established fact. In this study a series of experiments were carried out which yielded results that are not in accordance with this theory. The action of sodium oxalate in inhibiting coagulation is relatively slow. Since the precipitation of Ca^{++} by soluble oxalates is a rapid reaction, the behavior of sodium oxalate cannot be adequately explained by its action of merely removing free calcium. It is more likely that it decalcifies a compound which is essential for the formation of thrombin.

Of particular importance is the discovery that the prothrombin fraction

which is adsorbed by tricalcium phosphate from oxalated plasma is not removed by this agent from citrated plasma. It appears fairly certain that the citrate radical combines with prothrombin and it is suggested that the prothrombin is thereby inactivated. While this is admittedly not unequivocally proved, it does challenge the explanation that sodium citrate exerts its anti-coagulant action by depressing the ionization of calcium. A study of the stability of the labile factor of the prothrombin complex indicates that decalcification is not complete at the citrate level required to stop coagulation completely.

The theory that ionized calcium is required for the activation of prothrombin has been sterile as an aid to unravel the riddle of coagulation and probably has actually led many investigators to treat calcium indifferently. With the evidence marshalled against the theory of the indispensability of ionized calcium, the burden of proof now rests on those who maintain the validity of the theory and it is for them to provide data to support it. This statement has no polemic intent. It is merely to stress the urgency of further study.

The finding that adsorbents such as tricalcium phosphate do not adsorb the prothrombin fraction (which has generally been regarded as the classical unitary prothrombin) from citrated plasma makes the interpretation of several recent studies exceedingly difficult. Lenggenhager's hypothesis (6) of the existence of a prothrombokin, which is presumably similar to a prothromboplastin, is based on work done on citrated plasma and adsorption with tricalcium phosphate. Recently Milstone (7) in an extended study similar to Lenggenhager's, prepared a fraction which he designates prothrombokinas from euglobulin obtained from citrated plasma. He treated a solution of the euglobulin with BaSO_4 , apparently to free it from prothrombin. In the light of the present findings, it seems highly probable that his preparation contained some of the prothrombin citrate which is non-adsorbable. To be certain the experiment should be repeated using euglobulin isolated from oxalated plasma. These statements should not be construed as critical evaluations of Lenggenhager's and Milstone's work. They are cited merely to point out the possible pitfalls in applying methods such as adsorption which yield successful results with oxalated plasma, but not with citrated plasma.

The influence of storage on prothrombin activity is another point in question. In oxalated blood a decrease of 50 per cent may occur in 24 hours. Yet Warner, DeGowin, and Seegers (25) found that the prothrombin during storage in citrated plasma decreased only gradually, and required 3 weeks to reach the 50 per cent level. The results recorded in Table V hold the answer to this discrepancy of results. In plasma containing 0.2 to 0.3 per cent sodium citrate, the amount used in blood for transfusion, the labile factor is stable and therefore no rapid decrease in prothrombin activity occurs, in contrast to the rapid fall in oxalated plasma.

SUMMARY

1. The widely accepted theory that calcium participates in the coagulation mechanism in the form of Ca^{++} and acts as a catalyst is not in accord with several important experimental findings:

(a) The anticoagulant action of sodium oxalate is much slower than the precipitation of ionized calcium as the oxalate salt.

(b) Sodium citrate begins to depress prothrombin activity at a concentration at which ionized calcium is still present. The inability of tricalcium phosphate to adsorb prothrombin from citrated plasma indicates that citrate forms a complex with prothrombin and it is postulated that prothrombin is thereby inactivated.

(c) In plasma which is decalcified, *i.e.* in which the Ca^{++} is markedly reduced, the labile factor of prothrombin rapidly decreases. A concentration of 0.01 M sodium citrate sufficient to inhibit coagulation does not depress Ca^{++} enough to cause diminution of the labile factor, whereas when the concentration is increased to 0.02 M the labile factor decreases as rapidly as in oxalated plasma.

2. It is postulated that calcium functions in coagulation not as Ca^{++} but as combined with a component which is part of the prothrombin complex that is not adsorbed by tricalcium phosphate. A concentration of sodium citrate just sufficient to inhibit coagulation is not enough to remove calcium from the essential prothrombin component. The primary anticoagulant action of sodium citrate is therefore not decalcification but antiprothrombic.

3. It has been shown that citrated plasma is basically different from oxalated plasma in several important aspects. Unless cognizance is taken of these differences, serious errors and misinterpretations of experimental findings may be made.

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PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

II. THE RELEASE OF THE VIRUS FROM THE BACTERIAL CELL

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The mechanism of the release of bacteriophage from its host has been one of the most controversial subjects in the study of bacterial viruses.

D'Herelle's experiments led him to conclude that phage was released on the lysis of the host, these "bursts" occurring at definite intervals. The picture given by d'Herelle (1) indicated a lag period of 30 minutes after the addition of the virus to the bacteria, after which a 6- to 60-fold increase in phage occurred in a quarter of an hour. The titer then remained approximately constant for another 30 minutes until another sudden increase occurred. Doerr and Grüniger (2), using a *coli* phage, found a tenfold increase each quarter of an hour until the limiting concentration of phage was approached.

Other workers, however, reported that the virus could reach very high titers without lysis of susceptible bacteria (3-9). The well known lysogenic strain of *megatherium* which continues to form phage and to multiply is further evidence that the virus may be released from the bacterial cell without lysis of the host (10).

In a very extensive study Krueger and Northrop (11), working with a phage for *S. aureus*, reported that phage was released continuously from the bacteria and did not cause lysis of the cell until a critical ratio of phage to bacteria was reached. This conclusion was confirmed by Clifton and Marrow (12) and by Lin (13).

Ellis and Delbrück (14), working with a *coli* phage and using plaque counts to determine phage, found that the bacterial virus was liberated in steps and considered that the steps corresponded to the lysis of the bacteria with the subsequent release of phage, thus confirming d'Herelle's original observations.

In discussing the earlier results of Krueger and Northrop (11), Ellis and Delbrück (14) stated that the method of assay used by Krueger and Northrop might have obscured the step-formation of phage. They also pointed out that in the early formation of phage, the lysis of the cells would be obscured since the lysed cells would represent only a small fraction of the total bacterial cells present. The lysed cells would not be detected until the cells undergoing lysis represented a large fraction of the total bacteria. Krueger and coworkers (15) have reexamined the staphylococcus system previously studied by Krueger and

Northrop (11) in which the virus seemed to be formed continuously. Krueger and coworkers found that this phage system also released the bacterial virus in steps.

It had been previously reported from this laboratory (16) that in Fildes' synthetic medium containing a small amount of hydrolyzed casein, *Staphylococcus muscae* phage may be released without cellular lysis. If the concentration of the casein is increased, the cells are lysed. Recently Maurer and Woolley (17) working with the *E. coli* system have demonstrated that the addition of apple pectin to a synthetic medium results in the release of phage without cellular lysis. It thus appears that by varying the composition of the medium, the bacterial virus may be released without lysis of the host.

A more detailed study of the release of the bacterial virus will be reported in this paper. It will be shown that (a) in veal infusion medium the increase in plaque count occurs in a step-wise manner and is correlated with the lysis of the cell; (b) in Fildes' synthetic medium (18) containing hydrolyzed casein, the phage is also released in steps although no visible lysis occurs. This last observation was confirmed by turbidity measurements, hanging drop slides of the infected bacteria, and finally by staining the bacteria and observing them under the microscope.

RESULTS

The Release of Phage in Veal Infusion Medium.—Fig. 1 shows the release of phage in veal infusion medium from multiple infected cells. It can be seen that the phage titer remains constant for 30 minutes and then begins to increase at which time the cells begin to lyse; practically all the phage is released after the cells begin to lyse. It will be noted that the cells do not begin to lyse until 40 minutes. This is in fairly good agreement with the time of the minimum latent period. The relationship between the time of lysis in a multiple infected cell and the beginning of increase in phage titer in the *S. muscae* system is therefore similar under the above conditions to the *E. coli* B system previously studied by Delbrück (19). One-step growth curves carried out in veal infusion medium with single infected cells give average phage yields per cell in fairly good agreement with the average yield of virus particles from multiple infected cells. The minimum latent period is also the same as multiple infected cells; that is, 30 minutes. These observations are in agreement with the experiments reported by Delbrück for the *coli* system (19). It should also be observed that under these conditions cells infected with phage do not multiply. This result is in agreement with the studies of Cohen and Anderson (20) on the *coli* system.

The Release of Phage in Synthetic Medium

One-Step Growth Curve.—From Fig. 2 it can be seen that there is a minimum latent period of 30 minutes in synthetic medium. This is the same as in the

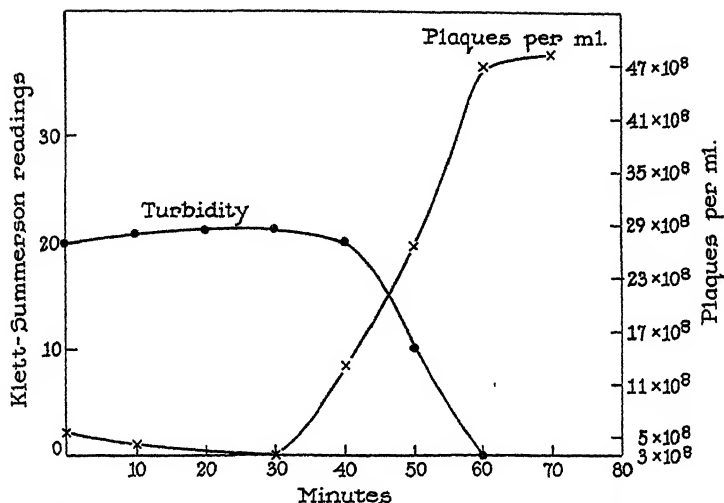


FIG. 1. Multiple infection in veal infusion medium. The bacteria were prepared as described under Methods. The bacteria were then added to 10.0 ml. of veal infusion medium and incubated 1 hour. After 1 hour the count was 1.1×10^8 cells per ml. Phage was added to give a final concentration of 5.2×10^8 particles per ml. The measurements were started. This experiment was run simultaneously with the experiment shown in Fig. 3 using the same bacterial and phage preparations.

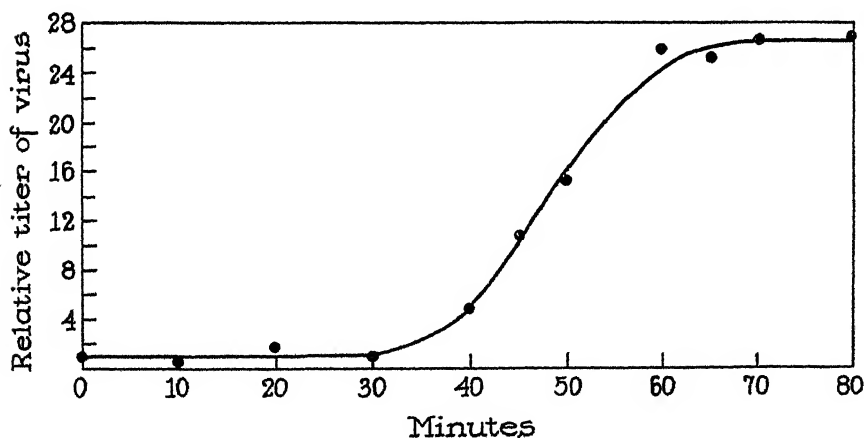


FIG. 2. One-step growth curve in synthetic medium. The cells were prepared as described under Methods. The synthetic medium, containing 10 mg. of hydrolyzed casein, was inoculated with 1×10^7 cells per ml. and allowed to incubate 3 hours. At this time the cell count was 5.1×10^7 cells per ml. 0.1 ml. of stock phage solution was added to give a final titer of 3.1×10^7 particles per ml. The tube was shaken for 18 minutes, and then diluted 1:2000 in synthetic medium containing 10 mg. of hydrolyzed casein. Centrifugation of the sample showed that 45 per cent of the virus was adsorbed. Samples were then taken from the diluted tube for phage assay.

veal infusion medium, although the cells grow more slowly in the synthetic medium. This observation is in agreement with the results obtained in the *coli* system (19).

Phage Release from Multiple Infected Cells.—Multiple infected cells in synthetic medium appear to release their phage in a step-wise manner before lysis of the cell. In the experiment shown in Fig. 3, there is a 20 minute interval between the lysis of the cell and the peak in phage formation. Plating out on agar showed that all the cells were infected after 30 minutes.

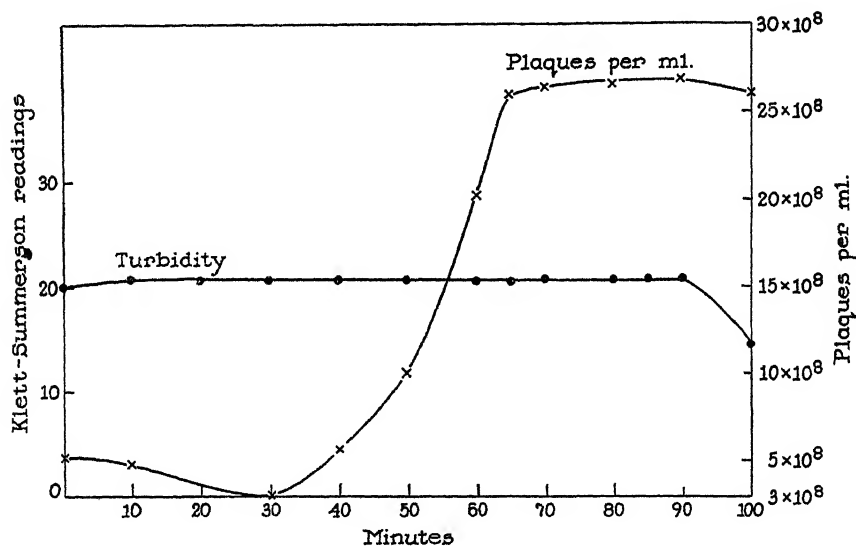


FIG. 3. Multiple infection in synthetic medium. The bacteria were prepared as described under Methods. The bacteria were then added to the synthetic medium. After incubation for 1 hour the cell count was 1.4×10^8 cells per ml. Phage was added to give a final concentration of 5.2×10^8 particles per ml. The measurements were then started. A control tube containing the same initial bacteria but no virus contained 3.2×10^8 cells per ml. at the end of 2 hours.

It may be argued that part of the cell is ruptured, but that the part remaining still gives the full turbidity reading. In order to check this point bacteria were removed from the tube and methylene blue smears made of the infected bacteria at various times before and after the phage had been released in a step. Many slides, containing many fields of infected bacteria were examined. Such slides, under oil immersion, showed that the bacteria had a normal morphology and no evidence of cellular rupture was seen even though all the virus was released. In order to account for the phage formed, all the cells would have to be ruptured, as pointed out below, and if this had occurred, such smears should have disclosed the ruptured cells. Finally, hanging drop slides showed the lysis of

the cells to occur at about the same time as the drop in turbidity and not at the time the step was completed.

In other experiments the difference in time between reaching the "step" and onset of lysis varied from 10 to 30 minutes. The time at which the "step" was reached varied from 60 to 82 minutes in different experiments. The minimum latent period also varied in some experiments appearing at 45 minutes instead of 30 minutes.

In several experiments the result shown in Fig. 4 was obtained. The step was finished at 72 minutes and the virus count then remained constant until

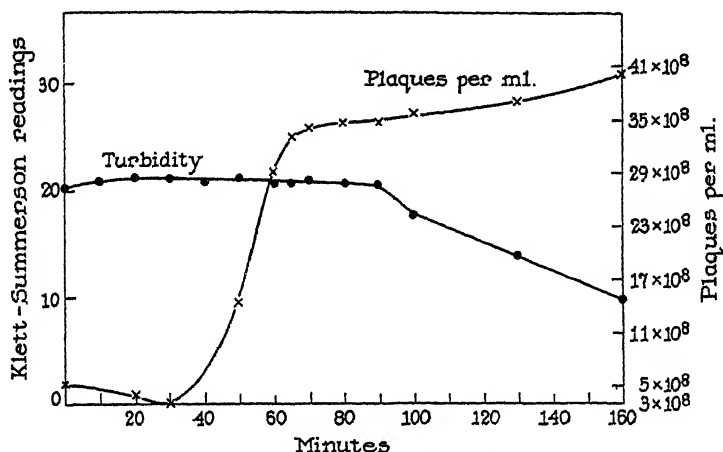


FIG. 4. Multiple infection in synthetic medium. The bacteria were prepared as described under Methods. 7.8×10^7 bacterial cells per ml. were added to the synthetic medium and allowed to incubate for 1 hour at which time the count was 1.0×10^8 cells per ml. Virus particles were then added to give a final concentration of 5.0×10^8 particles per ml. The measurements were then started.

90 minutes at which time lysis started. Phage counts during this lysis period showed a very slow rise in titer with lysis of the cell. This slow increase in phage titer is what one would expect since the plaque count method only determines infective centers. Some of the phage released in the step formation, which occurred in 72 minutes, would be adsorbed on bacteria. These bacteria would only give rise to one plaque even though they may contain two or more phages. As the cells were lysed from "without," however, the bacteria would release the adsorbed particles and then increase the titer.

These experiments seem to indicate two facts: (1) that phage may be released without any observable lysis of the cell and (2) that the step formation of phage need not necessarily be due to the lysis of the cell, since under the above condition of non-lysis, there is still a step formation of phage. It does not appear that this system is a lysis-inhibitor system such as that recently re-

ported by Doermann (21) for the following reasons: (a) phage release is correlated with cell lysis in the veal infusion medium, (b) there is no observable lysis corresponding to the release of phage as there is in Doermann's *coli* system, and (c) there are none of the other characteristics of Doermann's system. It does not appear likely that the phage is formed by a few lysed cells since a one-step growth curve in single infected cells carried out at the same time as the experiment in Fig. 3 showed that the average yield of virus particles liberated per cell was 22. To reach a titer of 2.5×10^9 virus particles per ml. as observed in the experiment shown in Fig. 3, there would have to be lysis of 1×10^8 bacteria per ml. if the phage produced is due to lysing cells. As the method of bacterial determination is accurate to 3 per cent (22), this could not have occurred, since there were only 1.0×10^8 bacterial cells to start with and no cellular lysis was observed until after the maximum virus titer was reached. It does not appear that lysis is obscured by multiplying cells since in this case the cells, when infected by phage, do not multiply and one is able to begin and end with the same number of cells and still reach the maximum phage titer. It should also be noted that the phage is released in this system and not contained within the cell. If the phage was not released, the plaque count method would not show any increase in titer, since this method only gives the titer of infective centers, as pointed out earlier.

DISCUSSION

The experiments reported in this paper indicate that the medium in which the experiment is carried out determines whether phage is released by lysis of the host cell or without visible lysis. In veal infusion medium the release of phage in *S. muscae* systems is correlated with the lysis of the host. Under these conditions, the release of the virus is very similar to that reported for the *E. coli* system (19). In Fildes' synthetic medium containing hydrolyzed casein, the results indicate that phage is released in a step-wise manner before visible lysis of the cell occurs. Under these latter conditions the disintegration of the cell may be due to "lysis from without." A comparable situation may exist in the *E. coli* system, since the release of phage is very similar in the two systems in a rich medium. Due to the greater capacity of *E. coli* to synthesize metabolites, it may not be possible to demonstrate phage release without lysis by the method used in this paper since the substance(s) involved in the lysis phenomenon may be synthesized by this host. Recently, however, Maurer and Woolley (17) found that the addition of apple pectin to a synthetic medium resulted in the release of *E. coli* phage without cellular lysis. This observation, together with the fact that phage may be formed in *E. coli* cells rendered non-viable by mustard gas (24), makes it possible to determine whether the virus is released in a step-wise manner in the *E. coli* system without cellular lysis.

The situation reported in this paper, in which the response of the host to a virus infection depends on the medium, may have its counterpart in the effect of various nutrient deficiencies on animal virus infections. Thus Elvehjem and coworkers (25) have shown that thiamine-deficient mice injected with Western equine encephalomyelitis do not show the typical pathological signs although the virus multiplied very well and killed the host. Other similar cases have also been reported (26).

It is interesting to speculate why the virus stops multiplying in the synthetic system before the cells begin to lyse. One interpretation could be that there is a substance in the cell essential for virus multiplication, which the virus-infected cell cannot synthesize, and virus formation stops when this compound is depleted. This hypothesis of limiting substrate could account for the great variation in virus yields per cell which has been observed by Delbrück (27) in the *coli* system, since cells could have varying amounts of the compound. In the *E. coli* system, furthermore, it seems probable that the yield per cell is also influenced by lysis, since Doermann (21) has shown that in lysis inhibition, the phage yield per cell is increased. If the virus synthesis does stop because of a depletion of some substance in the cell, attempting to prolong the increase in phage by adding various bacterial fractions at the time the "step" normally stops in the synthetic medium would be of interest. Another way of approaching the problem would be an analysis of the cell before and after the phage is formed. If the cells are centrifuged out from such systems, the phage inactivated with acid, and new phage and cells added to the medium, there is a good increase in phage. This result indicates that the virus does not stop multiplying because of some deficiency in the medium.

Another point of interest raised by these experiments is the explanation of the minimum latent period in phage formation. If one assumed lysis of the cell, the explanation of the minimum latent period was easy to understand. However, when the virus is released without lysis, the mechanism is difficult to visualize in view of Doermann's results that the phage has increased in the cell before it begins to be released. Why the minimum latent period has such a great time constancy under conditions of non-lysis must await future investigation.

The writer was assisted in this work by Mr. Mortimer Litovchick.

SUMMARY

1. The release of *S. muscae* phage in veal infusion medium is correlated with lysis of the host.
2. The release of the bacterial virus in Fildes' synthetic medium occurs in a step-wise manner before observable lysis of the cells occurs. This result has been confirmed by both turbidimetric readings and direct microscopic examination of the infected cells.

Methods

Bacteria were grown on agar slants for 20 hours as described previously (22). The bacterial cells were washed off with water, centrifuged, washed once with water, and then suspended in water before being added to the various systems. Phage and bacteria were determined as described previously (22). The phage used in this work was prepared in the synthetic medium described below to which was added 20 mg. of hydrolyzed casein. After complete lysis of the cells, the bacterial debris was centrifuged out at 5000 R.P.M. for 5 minutes. Such centrifuged lysates had a titer of approximately 4 to 9×10^8 plaques per ml. All phage dilutions were made in sterile distilled water.

Medium.—The veal infusion medium was prepared as described previously (22). The synthetic medium used was that of Fildes (18) which consisted of 6.0 ml. of amino acid mixture, 0.25 ml. of M/2 glucose, 0.25 ml. of M/500 ferrous ammonium sulfate, 0.10 ml. (100 γ) of nicotinic acid, 0.10 ml. (100 γ) of thiamine, 0.10 ml. of M/60 magnesium sulfate, 0.20 ml. of M/100 cystine, 0.20 ml. of M/100 methionine, 0.10 ml. of M/5 sodium nitrate, and 0.10 ml. of M/1000 tryptophane. To this medium was added 0.02 ml. of vitamin-free casein hydrolysate (equivalent to 2.0 mg. of casein) from General Biochemicals, Inc. To this solution was added the bacteria and phage. After all these additions, the solution was made up to 10.0 ml. with water.

Incubation of Reaction Mixtures.—All tubes were shaken at 36°C. as described previously (22).

One-Step Growth Curves.—The procedure of Delbrück and Luria (23) was followed.

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PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

III. THE COMPETITION BETWEEN HOST AND VIRUS FOR A NUTRIENT

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(Received for publication, June 10, 1948)

It has previously been reported from this laboratory that there is a substance present in acid-hydrolyzed proteins and yeast extract which is essential for the formation of *Staphylococcus muscae* phage (1, 2). Earlier experiments carried out in veal infusion medium indicated that there existed a competition for this nutrient between the host and virus (1). There also appeared to exist a competition effect between the host and virus for niacin (1).

With such a complex medium as veal infusion it was difficult to analyze the "competition effect" further, since it was impossible to control the amount of nutrient available. Different lots of veal infusion also had varying amounts of the nutrient. In order to study this system, it was desirable to find a synthetic medium whereby one could control all additions.

An analysis of the "competition effect" for the hydrolyzed protein factor has been carried out in synthetic medium and is reported in this paper. The results show that the casein factor is essential for phage production but not for the growth of the host cells, although growing cells may remove this factor from the culture medium.

RESULTS

Fig. 1 shows the effect of 10 mg. and of 2.0 mg. of hydrolyzed casein on phage formation. With the large amount of casein there is a steady increase in phage which finally results in cellular lysis. With the small amount of casein there is an increase in virus formation for the first 3 hours and then the increase of phage stops although the cells keep on multiplying. It appears that some substance is used up in the medium which is essential for the formation of the virus but not for the host, although the host may metabolize it.

Fig. 2 shows that the addition of hydrolyzed casein to a system in which the phage formation had stopped results in a further increase in virus. This experiment indicates that it is a factor in the hydrolyzed casein which is limiting the formation of the virus. This factor has been purified and its isolation will be described in a later publication.

A further analysis of the effect of a small amount of casein factor may be carried out by means of the one-step growth curve method. The results of such an experiment are shown in Table I. The cells were allowed to grow for

3½ hours to metabolize the small amount of casein present, since from Figs. 1 and 2 it can be seen that at this time the formation of virus stopped. At the end of 3½ hours phage was added to both tubes and more casein to only one tube. The adsorption of virus was the same in both samples but the cells which received the added casein formed 15 particles per cell, while the cells which did not receive casein formed only 2 virus particles per cell.

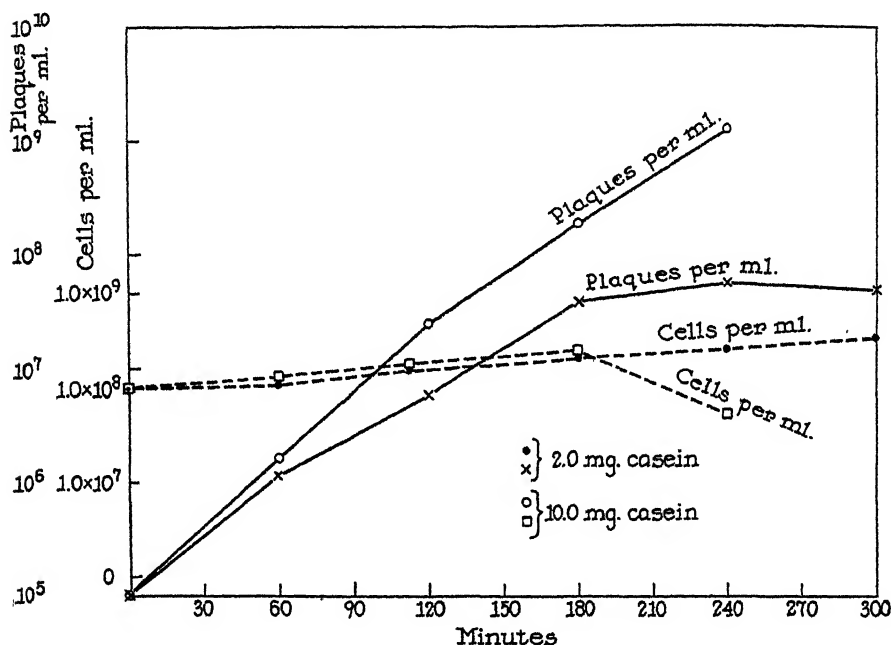


FIG. 1. The effect of varying concentrations of hydrolyzed casein on phage formation. The bacteria and phage were prepared as described under Methods. One tube received 2.0 mg. of hydrolyzed casein and the other tube 10.0 mg. of hydrolyzed casein. Bacteria and phage were then determined at intervals.

Fig. 3 illustrates the effect of varying the phage concentration in the presence of a small amount of casein. With the low concentration of phage only a few host cells are initially infected. These infected cells produce phage but in the meantime the non-infected cells use up the casein factor so that phage formation stops before all the cells are infected. With high phage concentrations many more of the host cells are initially infected, so that the host cells do not have the opportunity to remove the casein factor before they are all infected and under these conditions enough phage is formed to destroy all the cells. This explanation predicts that many of the host cells which continue to grow in the low phage tube are not infected. This prediction may be verified

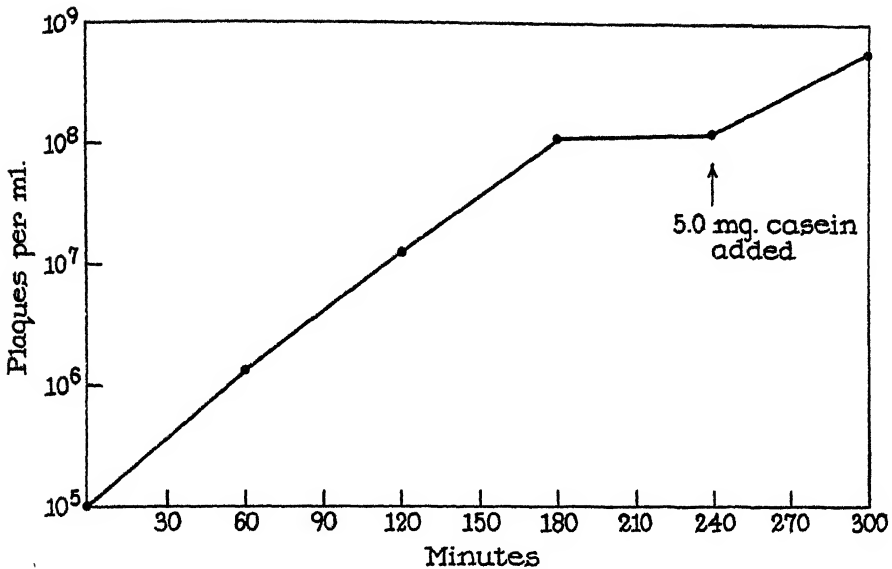


FIG. 2. The effect of adding hydrolyzed casein to a limited phage-forming medium. The same conditions were used as described in Fig. 1, with initial concentrations of 1.0×10^8 cells per ml., 1.0×10^6 phage particles per ml., and 2.0 mg. of hydrolyzed casein. Phage was determined every hour. At the end of 4 hours 5.0 mg. of hydrolyzed casein was added to the tube and phage determined at the 5th hour.

TABLE I

One-Step Growth Curve after Preliminary Incubation of the Cells

Eight tubes were set up each containing 10.0 ml. of synthetic medium plus 2.0 mg. of hydrolyzed casein. All tubes were inoculated with 7.1×10^7 bacteria per ml. and incubated $3\frac{1}{2}$ hours. At this time the cell count was approximately 2.7×10^8 cells per ml. in all tubes. Two tubes, A and B, were then inoculated with 0.2 ml. of phage to give 1.1×10^8 virus particles per ml. Tube B received 10.0 mg. of hydrolyzed casein. Tubes A and B were then shaken for 18 minutes, while the 6 remaining tubes were centrifuged 10 minutes at 5000 R.P.M. and the supernatant fluid saved. At the end of the 18 minute shaking period tubes A and B were diluted 1:10,000 with the supernatant fluid from the 6 centrifuged tubes. After diluting tubes A and B 1:10,000, tube B received 10.0 mg. of hydrolyzed casein, since the first 10.0 mg. of casein that was added was diluted out. A one-step growth curve was then carried out on the diluted samples of tubes A and B.

Sample	Casein added at $3\frac{1}{2}$ hours	Adsorption of virus to cells	Constant period	Rise period	Average yield of virus particles per cell
	mg./ml.	per cent	min.	min.	
A	—	36.5	30	30	2
B	1.0	35.1	30	30	15

by plating out such cells on broth agar after they have been centrifuged and washed. If the cells were infected, no colonies would form since infected cells undergo lysis on this medium. Actually, approximately 1.5×10^8 colonies per ml. were found. Subcultures from these colonies lyse if infected with phage in broth medium, showing that the cells are not a resistant strain. The continued growth observed in the presence of low initial phage concentrations

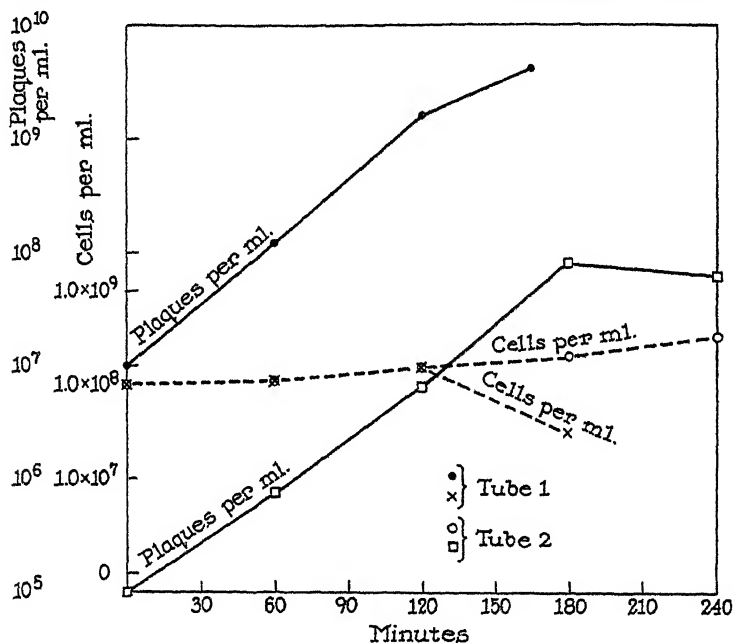


FIG. 3. The effect of a high and low initial phage concentration on the host cells. The same conditions were used as described in Fig. 1 except that 1.1×10^7 and 1.1×10^6 virus particles per ml. were added initially to tubes 1 and 2. The initial cell count was 1.0×10^8 cells per ml. and 2.0 mg. of hydrolyzed casein was added to each tube at the start of the experiment. Bacteria and phage were then determined at intervals.

therefore is not due to continual phage production with cellular multiplication as is found in lysogenic cultures but simply to the fact that the casein factor which is necessary for phage production has been used up.

DISCUSSION

The experiments reported in this paper confirm the earlier reported findings of the competition between the bacteria and virus for a substance present in yeast extract and hydrolyzed casein (1).

The results from these experiments indicate that the concentration of host cells, virus, and nutrient determines, to a certain extent, the over-all picture

one gets of virus formation. By varying the concentration of the above three components, it is possible to control the amount of virus formed, even to the extent of stopping and starting virus formation. The concentration of these three components also determines whether the virus destroys all the cells present in the test system.

There are several other points that should be emphasized in this work. The most obvious one is that the one-step growth curve should not be the only method used to study phage formation. If only a one-step growth curve had been carried out in the present work, the competition effect would have been overlooked. It was only by using a much higher initial concentration of bacteria than virus and then following the phage count continuously that the competition effect was observed. Once the effect was found, however, a system could be set up so that the result could be confirmed by a one-step curve.

The one-step growth curve method has the further serious disadvantage that it does not furnish information as to the effect of the changed conditions on the growth rate of the host cells. The results of many experiments show that phage production is closely associated with the growth of the normal host culture, in spite of the fact that infected cells do not grow. For example, if virus-infected bacteria were inoculated into different media, the medium which would best support the multiplication of the normal cells would in all probability give the best phage yields. It is only under very special conditions (3-6) that phage multiplication may be obtained in a medium which will not support the growth of normal host cells. Experiments such as those reported by Fowler and Cohen (7), which showed that the addition of certain nutrients increased the formation of *E. coli* virus, are difficult to interpret since the effect of the nutrients on the rate of growth of normal host cells was not determined. It is not possible to say, therefore, whether the observed effect on phage production is a direct one, or whether it is due to changes in the growth rate of the host cells. In attempting to study such systems, it is of course desirable to have a host which does not have too great a synthetic capacity.

From the present results it appears that the casein factor must be present in the medium at the same time as the virus if the virus is to utilize it for its formation. If the cell first metabolizes it and then the virus is added, it does not appear to be available for phage formation. One interpretation of this result could be that the casein factor is not synthesized by the bacteria and thus must be added to the medium for the virus to multiply.

In carrying out experiments on the nutritional requirements for phage formation, care should be taken not to carry over cells from one system to another. For example, the system used by Spizizen to study the nutritional requirements of phage formation is not an ideal one. Spizizen (8) permitted virus-infected bacteria to remain in broth for 7 minutes and then suspended them in buffer. Various compounds were then added and their effect on virus formation studied.

These results could be interpreted to mean that the nutritional requirements for virus formation were very few. This interpretation may not actually be correct, however, since in the short duration of the experiment, the nutrients taken up by the virus-infected bacteria in the first 7 minutes may have played a major rôle in virus formation. Under Spizizen's conditions the nutritional requirements for phage formation would therefore appear less than they actually were.

The results in this paper add another example to the many times shown relationship between nutrition and infectious disease (9). A very similar case to the one reported in this paper may be in the increased susceptibility of mice to pneumonia when they are fed a diet of natural foodstuffs instead of a synthetic one (10). Hitchings and Falco have recently purified this factor and have shown that it greatly intensifies the pneumococcal infection when given to mice (11). They have furthermore reported that extracts of these foods are capable of stimulating the rate of growth of pneumococci *in vitro*. This factor is not required by the mice. They conclude that "the unknown factor of the crude foodstuff is more beneficial to the parasitic organism than the animal consuming the food" (12). It is to be hoped that future studies will reveal similar examples, for analogues of these compounds may be helpful in the control of infectious disease. Analogues of this type, rather than of vitamins or other substances required by the host, have the possibility of being better chemotherapeutic agents since the host can do without the compound, and there is less of a chance that the analogue will interfere with an essential reaction of the host.

SUMMARY

1. Experiments carried out in Fildes' synthetic medium show that there is a competition between the host and virus for a substance present in acid-hydrolyzed casein. This substance appears to be essential for the multiplication of the virus but not for the host.

Methods

Bacteria.—Bacteria were grown on veal infusion agar slants for 20 hours at 37°C. The slants were washed off with 25.0 ml. of veal infusion and incubated 1 hour at 37°C. They were then centrifuged 5 minutes at 5000 R.P.M., washed once with water, recentrifuged, and suspended in water. 0.1 to 0.2 ml. of this suspension was added in all experiments to give the required cell concentration. The cells were allowed to incubate 1 hour in the synthetic medium before the experiment was started. Cells were determined as reported previously (4).

Synthetic Medium.—The synthetic medium, with varying amounts of hydrolyzed casein, was described earlier (2).

Phage.—Phage was prepared in synthetic medium as in earlier work (13). The supernatant fluid resulting from the 5000 R.P.M. centrifugation was centrifuged 1 hour

at 10,000 R.P.M. The precipitate, which contained the virus activity, was suspended in water. 0.2 ml. of phage suspension containing the required number of virus particles was added in all experiments. Phage was determined as in earlier work (4). One-step growth curves were carried out according to Delbrück and Luria (14).

Reaction Mixtures.—All reaction mixtures were shaken at 36°C. as in previous work (4).

Hydrolyzed Casein.—Vitamin-free acid hydrolyzed casein from General Biochemical, Inc., was used in all the experiments reported in this paper. It should be emphasized that various lots of hydrolyzed casein have varying amounts of the factor required for virus formation. It is therefore essential to titrate each lot of hydrolyzed casein in order to add the correct amount to demonstrate the competition effect. In all lots of hydrolyzed casein that we have worked with, it was possible to demonstrate the competition effect.

The writer was assisted in this work by Mr. M. Litovchick.

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INACTIVATION OF VIRUSES AND CELLS BY MUSTARD GAS

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Recent studies have shown that the sulfur and nitrogen mustards are unique reagents of considerable fundamental and practical interest. Thus it has been reported that (a) these compounds induce mutations in a variety of organisms (1-9), (b) that the carboxyl groups of many enzymes and proteins combine with the agents resulting in a decrease in enzymatic activity (10), (c) that several different animal viruses are inactivated by the sulfur mustard and the inactive preparations have vaccine properties (11), and (d) that preliminary reports indicate some hope for these agents being of therapeutic value (12-14, 50).

In the present communication it will be shown that viruses in general are particularly susceptible to the action of mustard gas (hereafter referred to as H). Following exposure to this agent, six animal, one plant, and two bacteria viruses, and the pneumococcus-transforming principle were inactivated at rates higher than the rates for the most sensitive enzymes but of about the same order of magnitude as noted for yeast and a number of bacteria.

Theoretical Considerations.—It was shown earlier (10) that the method of treating proteins with H did not affect the qualitative nature of the reaction. It was noted, however, that the extent of the inactivation of enzymes following a single exposure to H (formerly referred to as the Dixon method and hereafter designated as the single method) could not be calculated from a derived equation. It did fit a simple reciprocal relationship obtained empirically. Results of the inactivation of viruses, on the other hand, fit the theoretical bimolecular equations for both methods; *i.e.*, single and stirring methods. The single method has some practical advantages over the stirring method when dealing with viruses, bacteria, etc. under sterile conditions and has therefore been used in most of the experiments in this paper. The derivation of the equation used in conjunction with the single method will now be given.

Research work carried out during the war led to the conclusion that the active principle of mustard gas is not the β -chloroethyl structure but a cyclic intermediate ethylene sulfonium ion formed when mustard reacts with water (15). Although for the sulfur mustard a cyclic intermediate product has never been isolated, it has been generally assumed that one exists for the reaction

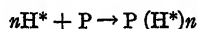
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properties of sulfur and nitrogen mustards are very similar and for the latter the intermediates have been clearly demonstrated (15, 16, 51).

The concentration of the active intermediate (hereafter referred to as H^*) is proportional to the H concentration or

$$[H^*] = C [H]$$

With the symbols P = protein, n = number of mols of H , t = time in minutes, and a = the rate of hydrolysis of H , then the reaction of H with any protein be it virus or enzyme may be written in the general form



and

$$\frac{-dP}{dt} = K[P][H^*]^n$$

$$\frac{-dP}{[P]} = K [H^*]^n dt$$

In the case where the $[H]$ is held constant throughout the experiment as in the stirring method, the integrated equation (see reference 10 for details) is

$$K = \frac{2.3}{[H]^n t} \log \frac{P_0}{P}$$

but since only H can be measured and not H^* , the equation becomes

$$KC = K' = \frac{2.3}{[H]^n t} \log \frac{P_0}{P} \quad (1)$$

In the single method the $[H]$ decreases logarithmically during the reaction or at any time t the $[H] = [H_0]e^{-at}$ and at time t , $[H^*] = C[H_0]e^{-at}$. Therefore the differential for the single method becomes

$$\frac{-dP}{dt} = K[P](C[H_0]e^{-at})^n$$

and

$$\frac{-dP}{[P]} = KC^n[H_0]^n e^{-ant} dt$$

upon integrating the following is obtained

$$-\ln P = \frac{-K(C[H_0]e^{-at})^n}{an} + B$$

where B is the integration constant. When $t = 0$ and $P = P_0$ then

$$B = \frac{K(C[H_0])^n}{an} - \ln P_0$$

Substituting this for B and rearranging:

$$\ln \frac{P_0}{P} = \frac{K(C[H_0])^n}{an} (1 - e^{-atn})$$

In both the previous and present experiments it was found that the inactivation rate did not vary as a higher power of the mustard concentration as predicted by the formula immediately above. Instead the results agreed with the assumption that $n = 1$ in spite of the fact that n , the number of H residues per mol protein, was actually greater than 1 as shown by direct analysis. A discussion of this apparent anomaly was given in the preceding paper (10). Since both the previous and present experiments clearly indicate that as far as the kinetics of the reaction with proteins is concerned $n = 1$, then the above equation simplifies to

$$\ln \frac{P_0}{P} = \frac{KC[H_0]}{a} (1 - e^{-at})$$

As t increases e^{-at} approaches zero. Therefore, if all samples are analyzed when t is relatively large (*i.e.*, after most of the H has reacted) the equation simplifies still further to

$$KC = K' = \frac{a}{[H_0]} \ln \frac{P_0}{P} \quad \text{or} \quad = \frac{2.3a}{[H_0]} \log \frac{P_0}{P} \quad (2)$$

It follows from Equation 2 that if measurements of virus activity are made after reaction with H is complete, the fraction of virus left will decrease logarithmically with increasing initial mustard concentration. It may also be predicted from Equation 2 that the rate or K' will be independent of the concentration of protein; *i.e.*, the same per cent inactivation will occur for a given H concentration regardless of the concentration of protein. These predictions are qualitatively confirmed by the results shown in Figs. 1-3.

The K' in Equation 2 is a velocity constant even though time does not appear

Details of Experiments Shown in Fig. 1

The Newcastle virus used in these experiments had been twice centrifuged but the E. E. E. was diluted allantoic fluid from an infected chick embryo. The solvent was water and the pH was maintained at 7.5-8.5 by the addition of dilute NaOH or bicarbonate as needed. The temperature was 25°C.

Details of the several methods are to be found in the section on Experimental methods.

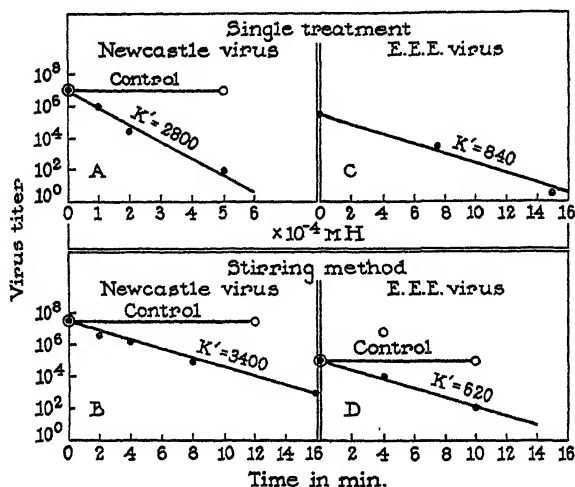


FIG. 1. Inactivation of Newcastle virus and equine encephalomyelitis virus by H.

Details of Experiments Shown in Fig. 2

The Type III transforming principle (TP) used in this experiment was kindly prepared and titrated by Dr. Rollin Hotchkiss and Dr. O. T. Avery following the current adaptation of the procedures previously described (33). To a series of 1 ml. aliquots of purified TP 54 containing 0.08 mg. nucleic acid in saline at pH 7.0-7.5 was added 1 ml. of saline solutions of H of various concentrations. Appropriate controls were included with hydrolyzed H and with saline alone. After standing 2 hours at 25°C. they were diluted and the activity of the residual TP determined.

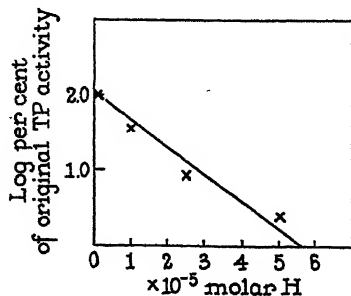


FIG. 2. Inactivation of purified Type III pneumococcus-transforming principle by varying concentrations of H.

Details of Experiments Shown in Fig. 3

The single method was used throughout this experiment. The pH was held between pH 7.5 and 8.5 with dilute NaOH or bicarbonate.

The experiment indicated in Fig. 3 by x was an allantoic fluid from an infected embryonated egg diluted one to five in water. In the experiment indicated by open circles o, the virus had been twice ultracentrifuged and resuspended in water. For the experiment represented by filled circles ●, the virus had been once ultracentrifuged.

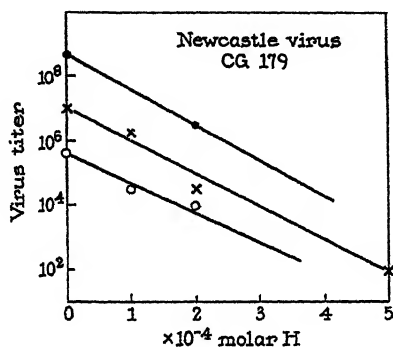


FIG. 3. Effect of the concentration of Newcastle virus on the rate of inactivation by H.

in the final integrated expression. The dimensions of the constant are: the fraction of P (protein or virus) reacting per mol of H per liter per minute.

EXPERIMENTAL RESULTS

Sensitivity of Various Biologically Active Materials to H

In Table I the sensitivity of various biologically active materials to H has been compared in two related ways. First, as the change in the logarithm of the biological activity per unit concentration of H and second, as the inactivation constants calculated with the aid of Equations 1 and 2. The first criterion is related to the second by the factor $2.3a$ for $\frac{\Delta \log P}{\Delta H_0} = \frac{K'}{2.3a}$ where a is the hydrolysis constant of H in the solvent used. Since the rate of reaction of H with any material including water is depressed by chloride ions, a comparison of rate constants in solvents of different chloride ion concentrations will involve this difficulty. Use of the direct measurement of the change in biological activity per unit H seems a preferable criterion of sensitivity to H for it is not dependent on the rate of reaction. This value also may vary with the chloride ion concentration as shown in Table I but the variation is specific for each material and independent of the effect on the rate of cyclization of H. It should be remembered in this connection that in the single method the reactions of H

Experimental Procedure for Table I

The solvent, temperature, and pH of the experiments analyzed and recorded in Table I are shown in the table. The pH was usually maintained with dilute bicarbonate or phosphate (*ca.*^m/1000).

In general unpurified preparations of the viruses were examined but as will be discussed later no appreciable difference in the inactivation is observed upon purification.

Three to five samples at different mustard concentrations (in the single method) and at different time intervals (in the stirring method) were removed and the biological activity determined in the manner described in the section on Experimental methods. The end point of a titration was the estimated concentration or dilution of biological agent which, in the case of viruses, produced death in 50 per cent of the five susceptible organisms used for each test. With cells, viability on broth-agar plates incubated for 48 hours at 37°C. was the criterion of inactivation. Phage was determined by plaque counts.

TABLE I
*Sensitivity of Certain Biologically Active Materials to Mustard Gas
at pH 8.0 ± 0.5 and 25°C.*

Material	Solvent	$\Delta \log \text{biological titer}^*$ $\Delta 1 \times 10^{-8}$ M H	K'	
			Single* method	Stirring method
Pneumococcus Type III transforming principle	Saline	30	2,800	
T ₂ r ⁺ phage of <i>E. coli</i> B	Water	20	5,600	
" " " "	Saline	6.3	600	
Newcastle virus (CG179 of Beach)	Water	12	3,500	4,000
" " (Beaudette)	"			3,500
<i>E. coli</i> B (d)	Locke's or saline	20	1,500	
" " " "	Water	10	2,800	
Swine plague bacillus	Broth and saline	9		
Feline pneumonitis virus (Baker)	Broth	9		
<i>Staphylococcus muscae</i> phage	Locke's	7	500	
Rabbit papilloma virus (Shope)	Water	6	1,700	
<i>E. coli</i> B (h)	Saline	5	500	
Equine encephalomyelitis virus (Eastern)	Water	3.6	1,000	800
<i>Staphylococcus muscae</i>	Locke's	3.6	250	
Rabbit myxoma virus	Saline	2.7	250	
Fixed rabies "	Water	2.5	700	
Bakers' yeast	M/10 NaCl	2.0		
" "	Water	0.8	220	
Tobacco mosaic virus	"	1.4	400	
Chicken pepsin	"	0.5	150	300
Crystalline yeast hexokinase	1 per cent glucose	0.2	ca. 65	

* These values have been calculated on the assumption that the change in the logarithm of biological titer varies linearly with changes in H concentrations. This is approximately true for most cases (Figs. 1-3). See the text for certain gross exceptions.

are allowed to go to completion before biological analyses are determined. Both methods of evaluating the sensitivity of biological materials to H are shown in Table I for various viruses,¹ cells, and a few enzymes for comparison.

In most instances the single treatment method of applying H was used but a few viruses were tested by both methods. In the single method several different mustard concentrations were applied to several equal aliquots of a given virus preparation and the logarithm of the remaining virus titer was plotted against the initial mustard concentration. The slope of such a plot is $\frac{\Delta \log P}{\Delta H_0}$

and equals $\frac{K'}{2.3a}$. Since a , the hydrolysis constant of H, is about 0.12 for water at 25°C. (16–20), 0.04 for saline, and 0.03 for Locke's solution, the value of K' is readily obtained. When the value of K' or the slope is known only a simple reversal of this calculation is necessary to find the change in virus titer that would be brought about by a given H concentration or to determine the H concentration necessary to reduce the virus titer by a certain value.

In Table I the solvent has been noted in each instance and just enough bicarbonate or phosphate was added to absorb the acid liberated by H and keep the acidity at pH 7.5–8.5. The temperature was 25°C.

The values in Table I for rabies, myxoma, papilloma, and swine bacillus were determined in each instance from only two experimental points per experiment though the experiments were repeated on several preparations of each agent. The minimum amount of H necessary to completely inactivate the disease agent and the initial titer are the two points on a log titer-H concentration plot from which the slope was obtained and the constants calculated. The values obtained in this manner are minimal since the concentration of H used to inactivate was presumably in excess. However, since the H concentration was varied by dilutions of two, the constants are low by less than a factor of 2 if the drop in titer with H concentration is linear with these viruses.² This assumption may prove to be incorrect but for many of the viruses no gross deviation was observed (Figs. 1–3).

It may be seen from Table I that the sensitivity to H of chicken pepsin, the

¹ The writer takes this opportunity to express his appreciation to a number of his colleagues who have made possible the examination of some of the most interesting material. Thanks are due Dr. Rollin Hotchkiss and Dr. O. T. Avery for their careful preparation and assays of the pneumococcus-transforming principle. I am also grateful to Dr. Carl TenBroeck for his titrations of the rabies virus and swine plague bacillus; to Dr. Margaret H. D. Smith for her preparation and titrations of the rabbit myxoma virus; and to Dr. Saul Malkiel for a number of preparations and titrations of tobacco mosaic virus.

² In the preparation of mustard vaccines in which the last trace of virus activity must be eliminated it is necessary to use somewhat more (1.5 to 2 times the mustard concentration) than that calculated from the values listed in Table I.

most sensitive enzyme examined in this laboratory, is considerably below all viruses and cells.

The values given in Table I are in general dependable within a factor of 2 and in some instances, depending on the virus and the number of points plotted, the reproducibility is better.

The pneumococcus-transforming principle is the most sensitive material yet analyzed. Its relationship to the general problem is discussed later.

In several instances the number of viable cells as determined by colony count did not decrease logarithmically as the mustard concentration increased. This was particularly true of *E. coli*. The constants for these systems were calculated from the average slope obtained during only the first 90 per cent inactivation. For reasons that are not apparent samples of *E. coli* B from two different laboratories have shown a marked difference in their sensitivity to mustard gas. A concentration of 1×10^{-4} molar mustard, which renders non-viable 50 per cent of the organism from one strain, similarly affects 99 to 99.9 per cent of the second strain; both having been grown in the same medium to the same concentration.

Constants calculated for the H inactivation of yeast from the figures reported by Kinsey and Grant (22) and by du Vigneaud and Stevens (21) are in agreement with the value in Table I.

It is of considerable interest that the cells were inactivated faster than the most sensitive enzyme and at about the same rate as found for the viruses. This is very suggestive that the sensitive locus of viruses and cells may have something in common. This bears further investigation particularly in view of the unique property of mustard of inducing mutations in cells such as *E. coli* (4). The criterion of "inactivated" or non-viable cells was their failure to grow on veal infusion peptone-agar plates on which normal cells grew luxuriantly. This does not mean that they could not have produced colonies on different media or evidenced growth under different conditions, although some recent experiments by the writer (52) render this less likely.

The work on cells shown in Table I and on a number of other organisms suggests that Gram-positive organisms are less susceptible to a given H concentration than are Gram-negative ones. More work is necessary to establish this but it is interesting in this connection that Henry and Stacey (23) have found that Gram-positive organisms have a coating of magnesium ribonucleate which is apparently absent in Gram-negative organisms.

Effect of the Method of Applying H on the Rate of Inactivation

Virtually the same inactivation constants are obtained by the stirring and single treatment methods of applying mustard to viruses as may be seen from Table I and Fig. 1. It should be noted in Fig. 1 that for the stirring method the abscissa is time whereas for the single method it is the initial H concentra-

tion. Only the constants K' are comparable. Details of the two methods are discussed later in this paper.

Effect of H Concentration on the Inactivation of Viruses

The results illustrated in Fig. 1 *A* and 1 *C* show how in the single treatment method the initial H concentration affects the extent of inactivation of two representative viruses. The straight line obtained by plotting the logarithm of the virus concentration remaining against the initial H concentration was expected if the derived Equation 2 correctly describes this system.

Fig. 2 shows the results of a similar experiment performed on highly purified Type III pneumococcus-transforming principle. This material exhibits even greater sensitivity to H than the viruses. Its relationship to the general problem is discussed later.

Chemical Nature of the Agent

It has been reported that in general the nitrogen mustards ethyl-bis(β -chloroethyl)amine (EBA), methyl-bis(β -chloroethyl)amine (MBA), and tris(β -chloroethyl)amine (TBA) have properties qualitatively similar to that of H (15). These three agents were tested on Newcastle virus and equine encephalomyelitis.

The nitrogen mustards as crystalline hydrochlorides were dissolved in cold water and then an aliquot added to a solution of virus buffered with bicarbonate just sufficiently to neutralize the acid liberated. Experiments showed that after a half hour at 25°C. there was not much change in the virus. This is in general agreement with the chemical studies of these agents (24).

It was found that whereas the $\frac{\Delta \log \text{biological activity}}{\Delta 10^{-3} \text{ M agent}}$ for EBA and MBA was not very different from H, for TBA it was two to three times higher; *i.e.* it required $\frac{1}{2}$ to $\frac{1}{3}$ as much TBA on a molar basis to obtain approximately the same inactivation.

Concentration of Virus

As was discussed in the previous paper (10), it follows from Equation 2 that the *fraction* of virus inactivated is independent of the virus concentration. Thus the slope of a plot of the logarithm of the virus concentration against the initial H concentration will be independent of the virus concentration. That this was observed may be seen in Fig. 3 where various initial concentrations of Newcastle virus were treated with various concentrations of H. All the curves have approximately the same slopes in agreement with the prediction.

Temperature

Temperature changes are known to affect markedly the rate of hydrolysis of mustard (17, 18). In an experiment with Newcastle virus it was observed

that the same inactivation per unit H was obtained at 0°C. as at 25°C. but it required many hours at the lower temperature for complete reaction compared to 30 minutes at 25°C. For the reader's convenience the values of the uni-molecular hydrolysis constant a for mustard in water at 0°C., 10°C., and 37°C. are about 0.004, 0.017, and 0.6 (17, 18).

pH

Whereas it had been found (10) with enzymes and other proteins that the rate of action was in some instances markedly affected by a change in pH of the

Details of Experiments of Table II

This experiment was carried out by the stirring method, described later in this paper, at both pH and with both viruses. The pH of the reaction mixture was examined continually with the aid of a Beckmann glass electrode and the acid liberated during the reaction was neutralized by careful addition of $m/100$ NaOH.

Samples were removed at 0, 1, 2, and 4 minutes for the Newcastle virus and 0, 4, and 10 minutes for the E. E. E. and the remaining activity determined by titrating on embryonated chicken eggs. The H concentration was 1×10^{-3} mols in all experiments.

TABLE II
Effect of pH on the Inactivation of Newcastle and Equine Encephalomyelitis Virus by Mustard

pH	Velocity constant K'	
	Newcastle	E. E.
6	5000	690
8	5000	620

medium, no such effect has been observed with the viruses of Newcastle disease and equine encephalomyelitis. The results in Table II carried out by the stirring method show that at pH 6 or pH 8 the inactivation constants of at least two viruses are the same.

Salt Concentration

Occasionally the reaction was carried out in saline solution instead of water. With certain of the viruses and most cells the results suggested that the presence of salt increased the degree of inactivation by H, while with other viruses there was no effect or it was in the opposite direction. Thus, equine encephalomyelitis, baker's yeast, and *E. coli* were inactivated more per unit H in the presence of $m/10$ NaCl or 0.85 per cent NaCl than in its absence, whereas *E. coli* phage was more sensitive in the absence of salt.

The hydrolysis or reactivity of H is retarded by the presence of chloride ion but by extending the time of contact between H and virus the reaction goes to completion.

Effect of Impurities or Other Reactants on the Rate of Inactivation

No consistent change in rate of inactivation could be demonstrated as the viruses of Newcastle disease or equine encephalomyelitis were purified by differential centrifugation or as the concentration of contaminating proteins varied. The three curves shown in Fig. 3 were obtained with an unfractionated Newcastle virus allantoic fluid, a once ultracentrifuged, and a twice ultracentrifuged sample. There is no appreciable difference in the inactivation rates of these three preparations.

The above conclusion received support when it was found that the extent of inactivation of pepsin by H was not markedly altered by the presence of ten times its weight of egg albumin. This should not be taken to mean that no impurities affect the rate of inactivation for it is well known that thiosulfate and similar substances have a high affinity for mustard and markedly affect the reaction. All halides slow down the reaction of H.

Comparison of the Effectiveness of H, Iodine, and Permanganate as Inactivating Agents of Newcastle Virus

It was of some interest to determine how mustard compared with other chemicals such as iodine and permanganate as inactivating agents. The results of an experiment are shown in Table III.

The amount of mustard combined is significantly less than the permanganate or iodine required to inactivate the virus. More mustard than is indicated in Table III was added to the virus solution to attain 90 per inactivation but from the nature of the reaction most of the H reacts with the solvent and only the amount indicated was bound to the protein.

Details of Experiments of Table III

A sample of twice ultracentrifuged Newcastle CG179 virus resuspended in water was mixed with sufficient reagent to make a final concentration in one tube of 1×10^{-4} M H, in another 1×10^{-5} M KMnO_4 , and in a third 1×10^{-5} M iodine. The virus concentration was 0.03 mg. protein per ml. After an hour at 25°C . the samples containing permanganate and iodine had lost their color. Samples were removed for virus activity tests in the usual manner. The iodine experiment was also carried out on a centrifuge-purified preparation containing 0.6 mg. virus protein per ml.

The KMnO_4 and iodine reactions were assumed to be stoichiometric. One experiment to test this point indicated that this was qualitatively true for iodine. The reaction of H, however, is known to consist of two simultaneous competing reactions in which the reaction with protein is a small fraction of that reacting with the water. To determine the H bound to the virus it was necessary to use a solution of twice centrifuged Newcastle virus containing 5.6 mg. of protein per ml. and an initial 50 per cent end point titer of 10^{11} . After treatment with 1×10^{-4} M H for $\frac{1}{2}$ hour at 25°C . which reduced the activity to 10 per cent of its original titer or to 10^{10} , this solution and an untreated virus control diluted to the same volume were angle centrifuged at 12,000 R.P.M. for an hour in a centrifuge of 9 cm. average radius. The uncombined H or its hydrolysis product in the supernatant was then titrated with bromine in acid solution using methyl red as the end point indicator (10). From this and the similar titrations of the appropriate controls, *i.e.* H solution plus no virus and virus supernatant when no H had been added, the value for the bound H was obtained. The value was very small and is the value shown in Table III. It should be emphasized that this is not an accurate determination for it was obtained as the difference between two close and large values. It does serve, however, as a qualitative upper limit of the amount of H combined.

TABLE III
Certain Chemical Agents on Newcastle Virus at pH 7-8 and 25°C .

Reagent	Reagent consumed or used up per milligram of purified Newcastle virus in inactivating (<i>ca.</i>) 90 per cent of the virus
	<i>mM</i>
Iodine	1×10^{-4}
KMnO_4	$>1 \times 10^{-4}$
H	$<0.03 \times 10^{-4}$

EXPERIMENTAL DETAILS

Materials and Biological Measurements

Equine Encephalomyelitis (Eastern Strain).—This virus was obtained from Dr. Carl TenBroeck of this laboratory. It was highly infectious for 10 day old chick embryos killing them in 24 to 48 hours. Allantoic fluids from embryos harvested after 23 hours' incubation at 37°C . usually contained 10^8 M.L.D. per ml. Titration of the virus followed the usual procedure (39). Serial tenfold dilutions of the virus were made in cold buffered (pH 7.4) saline. A drop (0.05 ml.) of the dilutions near the

expected end point was placed on the chorioallantoic membranes of 10 day old embryonated eggs after which they were incubated at 37°C. and examined daily with the aid of a candling lamp. All dead embryos were examined and pale uncongested ones were not counted as indicative of the presence of virus. Five embryos for each sample at each dilution were used. The virus titer was estimated as the concentration which would kill 50 per cent of the embryos. In our hands the results were reproducible to at least 0.5 logarithm units or about three times in the virus concentration.

Newcastle Disease of Chickens

Beaudette Strain.—This strain was obtained from Dr. F. B. Bang who in turn obtained it directly from Dr. Beaudette of the New Jersey Experiment Station, Rutgers Agricultural School. This strain kills embryos in 36 to 72 hours but in general does not kill adult birds. Allantoic fluid from embryos inoculated with 10^8 M.L.D. and subsequently incubated for 44 hours usually contained 10^8 or more M.L.D. per ml. Very few studies were made with this strain.

CG179 Strain of Beach

This strain which kills both embryos and adult birds was obtained from Dr. Carl TenBroeck who in turn obtained it from Dr. J. R. Beach in California. After inoculation with 10^5 to 10^6 M.L.D., allantoic fluids from eggs maintained at 37°C. for 38 hours usually contained 10^8 M.L.D. This strain kills embryos a little faster than the Beaudette strain. Practically all the studies on Newcastle virus in the present paper were made on this strain. Titration of the virus followed the same procedures described for equine encephalomyelitis except that the eggs were examined several days longer.

E. coli B

Most of our work was done on an organism kindly sent to W. H. Price by Professor M. Delbrück. Toward the end of our work we had reason to request a sample of *E. coli B* from Professor A. D. Hershey. We were considerably surprised to find that it exhibited greater resistance to the action of H than did the sample from Professor Delbrück. Both organisms are lysed by T_1 , T_6 , and T_7 phages in the same time. No other phages were available for test at this time.

We have indicated in Table I the organism from Delbrück as (*d*) and from Hershey as (*h*).

Culture of this organism was in the usual broth media. Plating consisted of making the final dilution in 0.7 per cent agar in broth at 43°C. and 1 ml. was spread on warm previously poured agar-broth plates. Dilutions of the organism were made in saline buffered at pH 7.3.

Staphylococcus muscae Phage

W. H. Price kindly furnished this bacterial virus and host. He obtained them from Dr. R. E. Shope (41). This phage was diluted in Locke's solution, then into 0.7 per cent agar-broth with 1×10^8 actively growing *Staphylococcus muscae* cells per ml. One ml. of this suspension was spread on an agar-broth plate and incubated overnight at 37°C. after which the plaques were counted.

Staphylococcus muscae

The procedure for determining these cells was the same as that described above for *E. coli* B except that dilutions were made in Locke's solution.

Bakers' Yeast

This was fresh Fleischmann's bakers' yeast transferred and grown in a synthetic medium consisting of 2 per cent sucrose, 0.3 per cent $(\text{NH}_4)_2\text{SO}_4$, 0.2 per cent KH_2PO_4 , 0.025 per cent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 per cent $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.1 per cent extract. Colony counts of 2 per cent agar media plates are quite reproducible. Incubation temperature was 30°C.

Feline Pneumonitis (Baker)

This agent, a member of the psittacosis group (42), was obtained from Dr. J. A. Baker (43). The virus was handled as previously described in the above references. Suspensions and dilutions were made in veal infusion broth. Inoculations of 1 ml. were made into the yolk sac of 5 day embryonated eggs. A 10 per cent yolk sac suspension made from eggs in which the embryos were moribund contained at least 10^8 M.L.D. for chick embryos. It was shown by Hamre and Rake (42) that the time of death as well as the dilution end point could be used as a rough measure of the virus concentration in the inoculums.

Rabbit Papilloma Virus (Shope)

This virus was obtained through the kindness of Dr. R. E. Shope and consisted of glycerinated cottontail horny papillomas. The virus was prepared and titrated as previously described (44).

*T₂^r Phage of *E. coli* B*

This bacterial virus and host were originally obtained by W. H. Price from Professor M. Delbrück. Estimation of the phage was made by mixing 1 ml. of his buffered saline dilution of the phage containing about 10^8 M.L.D. per ml. with 9 ml. of an 0.7 per cent agar-broth mixture in which were also suspended about 3×10^7 actively multiplying *E. coli* B cells per ml. One ml. of this mixture was then spread on a broth-2 per cent agar plate and left at 37°C. overnight after which a count was made.

Crystalline Yeast Hexokinase

This purified protein was obtained from Dr. M. Kunitz and estimated as described by Kunitz and McDonald (45). In our previous paper (10) we indicated that the action of H on hexokinase was not examined at pH 7.5-8 because it was too unstable under these conditions. One per cent glucose stabilized the enzyme at this pH sufficiently to perform the experiment. As seen in Table I the rate of inactivation was still considerably lower than found for chicken pepsin or any of the viruses.

Chicken Pepsin

This enzyme was obtained as previously described (46). The activity was measured by its milk-clotting property (47).

Sulfur Mustard Gas

Most of the work reported herein was done with mustard prepared from thiodiglycol. It had a freezing point of 14.2 to 13.8°C., the change in melting point occurring over a long period. An experiment was performed on Newcastle virus with the above preparation and a highly purified preparation supplied by Dr. V. du Vigneaud (21) (m.p. 14.5°C.) and they showed the same degree of inactivation. Other experiments were performed in which *E. coli* B (d) and the T₂r⁺ phage were treated with four different preparations of H the melting points of which varied from 12°C. to 14.5°C. All showed virtually equal effect on the biological agents. It may be concluded, therefore, that the impurities present in these preparations did not inhibit the biological test as was noted for yeast by du Vigneaud and Stevens (21) when certain of their impure preparations were used.

The Nitrogen Mustards

All three of these materials as crystalline hydrochloride salts were obtained from Edgewood Arsenal.

Aqueous Solutions of Mustard

Aqueous solutions of mustard were always made up fresh when they were to be used. A convenient method was to add 0.01 ml. of liquid H to 80 ml. of cold water or saline in a glass stoppered bottle and immediately shake hard for 15 to 30 seconds. Care must be taken to shake immediately after addition of the H to cold water for otherwise it may crystallize and the time required to dissolve the crystals has not been determined but it is probably considerably longer. Complete solution of the H results in a 1.0×10^{-3} molar solution. If the temperature is 10°C. or below, it will not hydrolyze perceptibly for several minutes. The concentration of H in water saturated at 5–10°C. is 4.5 to 5.0×10^{-3} molar. For other data in this connection one may find another paper (48) of interest.

If it is desirable the H can be first diluted in an organic solvent which is miscible with water such as alcohol or one of the "cellosolves." Such a solution permits a larger volume to be measured and mixed with water for the preparation of homogeneous solutions. Unless the aqueous solution is agitated as the organic solution of H is added, the H will precipitate locally and may not dissolve unless shaken hard.

General Methods

Determination of Mustard Concentration in Solution

Methods for differentiating free H from sulfonium salts and thiodiglycol are described elsewhere (10, 49). The basic titration is with bromine or hypochlorite in $m/4$ H₂SO₄ with methyl red as an indicator (10).

Methods of Exposing Materials to H

Single Method

This simple method consists of making an aqueous or saline solution of H and mixing in the desired proportions with the virus or biological material. Preparation of aqueous H solutions has been described in an earlier paragraph.

After mixing with the biological material the container was kept at 25°C. for at least 30 minutes if no salt was present but 60 minutes if 0.85 per cent saline was used.

Stirring Method

A variation in the previously described stirring method was used in the present experiment. In the earlier paper excess free H was stirred and a constant concentration of H resulted when an equilibrium was obtained between the rate of solution and the loss due to hydrolysis. In the present procedure the amount of H to give the desired concentration was first dissolved in the solvent (water or saline) in the usual way. The biological agent was then added and then the concentration of H maintained constant by permitting a "cellosolve"³ solution of H to flow in very slowly through a fine capillary while the aqueous solution was stirred. By adjusting the initial volume of aqueous solution, the diameter and length of the capillary, and the concentration of H in the "cellosolve," any concentration in an aqueous solution can be maintained. When the concentration of "cellosolve" interferes with the reaction or the biological agents then this procedure will have to be modified. The amounts of "cellosolve" introduced in the present experiments were usually 0.05 to 0.10 ml. per minute and this was found to have little if any effect on the viruses studied.

In this method small samples are removed at intervals of time and diluted 10 to 100 times with cold buffered saline which virtually stops the inactivation by H. After dilution the activity of the sample was determined in the appropriate manner for the particular material.

The changes in pH were followed by means of external glass electrodes connected to a Beckmann pH meter. Corrections were made by permitting small quantities of dilute NaOH or bicarbonate to flow in through a capillary.

The apparatus was sterilized before use with phenol or 70 per cent alcohol followed by numerous washings and soaking with sterile water.

DISCUSSION

The obvious difference between viruses and enzymes that might account for the consistently greater susceptibility of the former to mustard is the nucleic acid present in viruses. In the past few years it has become well established that mustard gas combines with nucleic acid, nucleoproteins, and viruses (25-30, 53). In most of these instances cited the quantity of mustard used and the length of exposure were such that the treatment is considered more drastic than that described in the present work. In addition there was no indication of the rate of reaction in these earlier studies. Gilman and Phillips (15) have reported that du Vigneaud *et al.* and Bawden and Pirie found that mustard inactivates tobacco mosaic virus but a detailed account of this work has not yet appeared. More recently TenBroeck and Herriott (11) showed that the animal viruses equine encephalomyelitis, fixed rabies, and hog cholera are inactivated by small amounts of mustard but no consideration was given at that time to the rate of the reaction. Rose and Gellhorn (30) have recently found that influenza virus is inactivated by H as well as the nitrogen mustards.

³ "Cellosolve" is a trade name for monoethyl ether of ethylene glycol.

The results of the present studies suggest that the viruses containing desoxyribose nucleic acid (DNA) are more rapidly inactivated than those containing only ribosenucleic acid (RNA). This may be seen in Table IV where certain chemical analyses are shown along with the relative sensitivity to mustard.

TABLE IV
Composition of Biologically Active Materials and Their Sensitivity to Mustard Gas

Material	$\Delta \log$ titer $\frac{\Delta 1 \times}{10^{-8} M H_2}$	Protein	Carbo- hydrate	Lipid	Nucleic acid		Refer- ence No.
					Desoxy- ribose-	Ribose-	
			per cent	per cent	per cent	per cent	
Pneumococcus Type III transforming principle	30				ca. 100		33
T ₂ r ⁺ phage of <i>E. coli</i> B	20	51	13.6	2	30-40		34
<i>E. coli</i> B	20	67.7		9.1	2.4	21	34
Newcastle virus	12	67		27	1		35
Papilloma	6*	90		1.5	8.7		36
E.E.E.	3.6	49	3.5	48.5		4.4	37
Tobacco mosaic	1.4	94		0	0	6	38

* This is a minimum value and may be higher. See text p. 227.

Considerable interest was added to the above relationship when it was found that the purified pneumococcus-transforming principle (TP) was the most sensitive material yet examined. The present evidence indicates that the preparations of TP are relatively pure DNA in which the presence of protein has not been demonstrable.

The finding of Mirsky and Ris (31) that the isolated chromosomal material from lymphocytes is extremely rich in desoxyribose nucleohistone is interesting in view of the repeated observations on a variety of organisms that mutations may be induced with mustard gas (1-9).

Bodenstein and Kondritzer (32) have recently reported that the formation of DNA in amphibian tissue stops following exposure to dilute H which also arrests mitosis. In direct contrast the RNA formation proceeds apparently in a normal manner.

All of the above independent observations are suggestive but the work in progress employing mustard in which radioactive sulfur has been incorporated should provide a definite answer to the obvious question of the location of the bound mustard.

SUMMARY

The action of mustard gas on six animal, one plant, and two bacterial viruses; also on bacteria, yeast, and the pneumococcus-transforming principle has been studied. The viruses include Newcastle's disease of chickens, equine encephalo-

myelitis (Eastern strain), feline pneumonitis (Baker), rabbit papilloma (Shope), fixed rabies, rabbit myxoma, tobacco mosaic, T_2r^+ phage of *E. coli* B, and a *Staphylococcus muscae* phage. The cells include bakers' yeast, *E. coli* B, *Staphylococcus muscae*, and swine plague bacillus.

The rates of inactivation of the viruses and cells were of the same order of magnitude and faster than those of enzymes.

Of the viruses examined those containing desoxyribose nucleic acid were inactivated faster than those containing ribonucleic acid. Preparations of the pneumococcus-transforming principle which were largely desoxyribose nucleic acid have shown the greatest sensitivity to mustard gas of all systems examined.

An expression was derived describing the inactivation rate when mustard gas decreases during the experiment.

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CRYSTALLIZATION OF SALT-FREE CHYMOTRYPSINOGEN AND CHYMOTRYPSIN FROM SOLUTION IN DILUTE ETHYL ALCOHOL

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The original methods developed for the isolation of crystalline chymotrypsinogen and chymotrypsin involve the use of ammonium sulfate (Kunitz and Northrop, 1935). It has recently been found possible to crystallize the purified preparations of chymotrypsinogen and chymotrypsin from dilute solutions of ethyl alcohol free of any salts. The crystals of both materials obtained in the presence of alcohol differ in form from those obtained in the presence of ammonium sulfate. The enzymatic properties of the new crystals, however, are identical with those of the crystals isolated from solution in the presence of ammonium sulfate.

Several enzymes and other proteins have recently been crystallized from solution in dilute alcohol in the presence or absence of salts.¹

Ribonuclease has been crystallized from salt-free 50 per cent alcohol (Kunitz, 1940).

Northrop (1946) reported the crystallization of pepsin from solution in 15 to 20 per cent alcohol in the presence of low concentrations of magnesium sulfate.

Soybean trypsin inhibitor and also the trypsin-soybean inhibitor compound were found to crystallize readily from salt-free 20 per cent alcohol (Kunitz, 1946, 1947).

Cohn, Hughes, and Weare (1947) employed alcohol in the presence of low concentrations of acetate buffer for the crystallization of human and bovine serum albumins.

The methods of crystallization of chymotrypsinogen and chymotrypsin from alcohol are as follows:—

1. Chymotrypsinogen

(a) *Preliminary Treatment.*—Chymotrypsinogen is isolated from fresh beef pancreas and is recrystallized several times in the presence of ammonium sulfate, as described in the original publication on the isolation of chymotrypsinogen (Kunitz and Northrop, 1935).

(b) *Removal of Ammonium Sulfate by Dialysis.*—Ten gm. of semidry filter-cake of crystals of chymotrypsinogen is stirred up with about 30 ml. of distilled water and is dissolved with the aid of several drops of 5 N sulfuric acid. The solution is then dialyzed in a collodion or cellophane bag against slowly running distilled water for 24 hours at about 5°C., preferably with stirring.

¹ Alcohol as an aid in crystallization of proteins was employed as early as 1882 by Hüffner and Otto (1882) for the isolation of methemoglobin.

(c) *Crystallization from Alcohol*.—The dialyzed solution of chymotrypsinogen is filtered clear and then made up with water to 50 ml. The pH of the solution is adjusted with dilute acid or alkali to about 4.0. The solution is cooled in an ice-salt bath to 1–3°C. and 12.5 ml. of ice cold 95 per cent alcohol is added slowly with stirring; the temperature of the solution is not allowed to rise above 5°C. during the addition of the alcohol. The pH of the solution is then adjusted with 1 N sodium hydroxide to about 5.0.² A heavy amorphous precipitate forms at pH 5.0. The suspension is kept at 20 to 25°C. The precipitate gradually dissolves and is replaced within several hours by a crop of large, well formed crystals shown in Fig. 1. The crystallization is generally complete within 24 hours. The crystals are filtered with suction on hardened paper, washed with ice cold acetone, and dried in the room for 24 hours. The dried material is ground up in a mortar to a fine powder and is stored in a refrigerator.

The dry powder dissolves readily in dilute sulfuric acid at pH about 3.0 and yields crystalline chymotrypsin when treated as described in the original publication (Kunitz and Northrop, 1935).

A heavy suspension of the dried preparation of chymotrypsinogen in water at pH 5.0 and 20°C. gradually begins to yield long needles³ similar to those formed on crystallization of chymotrypsinogen in the presence of ammonium sulfate. The formation of the needles is readily demonstrable by preparing on a microscope slide under a cover slip a small drop of a thick suspension of the dry crystals in water. The needles begin to appear within 2 to 3 minutes and the progress of the formation and the growth of the needle crystals can thus be observed through a microscope.

2. Chymotrypsin

(a) *Preliminary Treatment*.—Chymotrypsin is prepared from crystalline chymotrypsinogen with the aid of trypsin as a catalyst, and is recrystallized once or twice in the presence of ammonium sulfate (Kunitz and Northrop, 1935).

(b) *Removal of Salt by Dialysis*.—Ten gm. of crystalline filter cake is dissolved in 30 ml. of cold water and then dialyzed against slowly running 0.005 N sulfuric acid at about 5°C. for about 24 hours, preferably with stirring.

(c) *Crystallization from Alcohol*.—The dialyzed solution is filtered clear, then made up with water to a volume of 50 ml., and cooled in an ice-salt bath to 2–3°C. The pH of the solution is adjusted to about 4.8 with the aid of several drops of 1 M sodium hydroxide. Ten ml. of ice cold 95 per cent alcohol is added slowly with stirring,

² The pH is tested by the drop method on a test plate using 0.1 M acetate buffer pH 5.0 as a standard and 0.01 per cent methyl red solution as an indicator.

³ The crystalline material on drying apparently changes into an amorphous material which evidently possesses a greater solubility in water at pH 5.0 than the needle crystals.

The crystallization of pure chymotrypsinogen in form of needles from a concentrated solution of the protein in water at pH 5.0, even in the absence of salt, has been recently observed and reported by Jacobsen (1947).

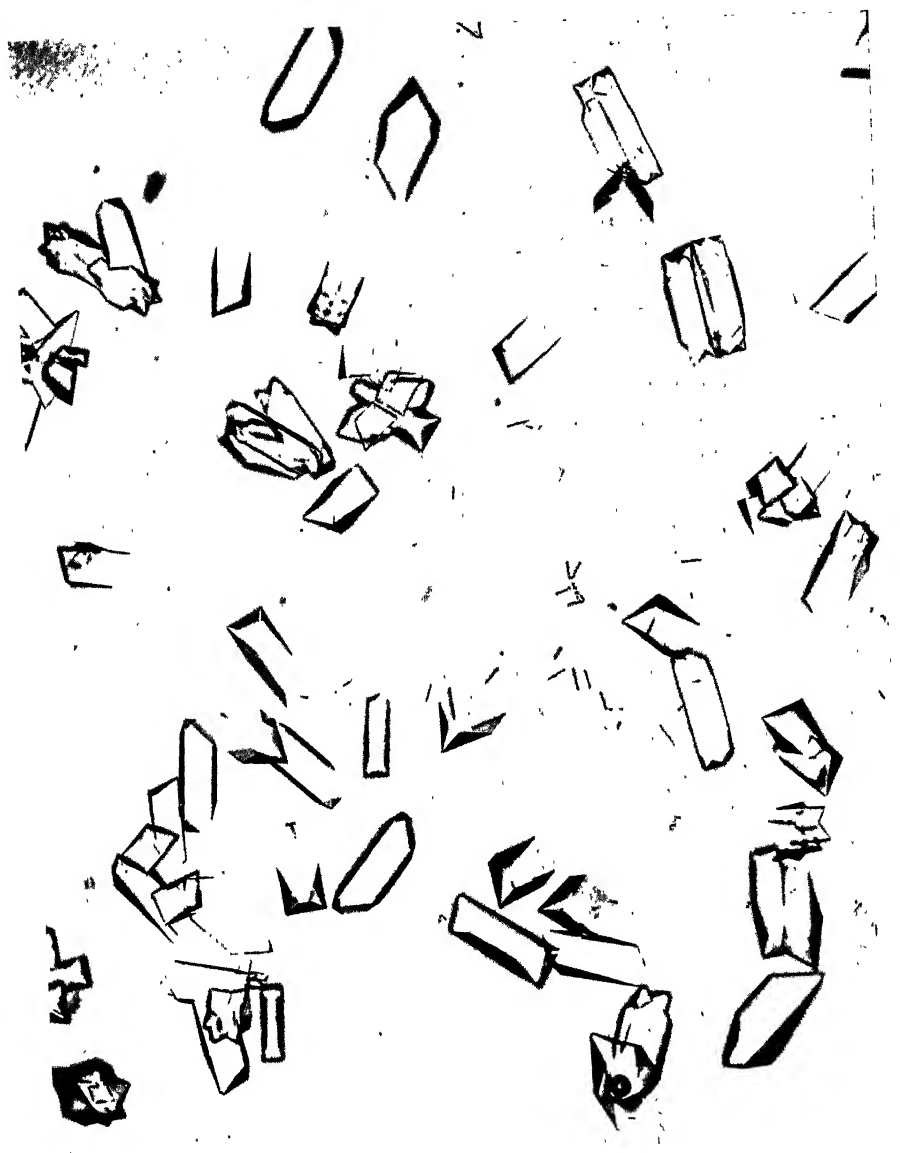


FIG. 1. Chymotrypsinogen crystals in dilute alcohol. $\times 117$

while the temperature of the solution is maintained at about 5°C . The solution is then titrated with 1 N sulfuric acid to pH 4.0 (light green to brom cresol green on test plate). A heavy amorphous precipitate is formed. The suspension is kept at about 5°C . The amorphous precipitate slowly changes into a paste of very fine needles and



FIG. 2. Chymotrypsin crystals in dilute alcohol. $\times 141$

rosettes (Fig. 2). Seeding with a drop of a suspension of the crystals assures prompt crystallization within 24 hours. The paste of crystals is filtered with suction on hardened paper at about 5°C . The filtration generally takes several hours. The filter cake is dried on a watch glass placed for several days near the cooling coil in an

electric or gas refrigerator. The dry glassy material is ground in a mortar to a fine powder and stored in the refrigerator.⁴

The dry material dissolves readily in water at pH 3 to 4. It yields the usual rhombohedrons when recrystallized in the presence of ammonium sulfate at pH below 5.0.

The protease and milk-clotting activity per milligram protein of the dry salt-free powder is identical with those of the preparations of chymotrypsin crystallized in the presence of ammonium sulfate.

SUMMARY

Chymotrypsinogen and chymotrypsin crystallize readily from dilute solutions of ethyl alcohol in the absence of salts. The crystals formed in the presence of alcohol differ in appearance from those formed in the presence of ammonium sulfate. Chymotrypsinogen yields well formed polyhedrons instead of fine needles usually produced in ammonium sulfate solution. Chymotrypsin yields fine needles in the presence of alcohol and rhombohedrons in the presence of ammonium sulfate. The enzymatic properties of the crystals formed in the presence of alcohol are identical with those of the crystals isolated in the presence of ammonium sulfate.

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⁴ The use of acetone as an aid in drying of the crystals is to be avoided because of the danger of partial denaturation of the relatively labile chymotrypsin.

TRACER STUDIES OF NITROGEN ASSIMILATION IN YEAST

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The work of Caspersson (1) and others on the intracellular distribution of nucleic acids in cells has led to the concept that ribonucleic acids are intimately associated with the synthesis of protein. Furthermore, some evidence has been accumulated indicating that not only the concentration of ribopolynucleotide but also the metabolic activity increases during active protein synthesis. Thus tracer studies with radioactive phosphorus have indicated a more rapid turnover of the phosphate component of the polynucleotide when protein was being laid down (2-4). In the present report we have used N^{15} as a tracer to observe the formation of protein and nucleic acid purines in yeast. This organism was selected because of its rapid growth rate, because the state of its nitrogen metabolism could be readily controlled (5), and because ultraviolet microscopy had indicated the importance of nucleic acid in growth (6, 7).

EXPERIMENTAL

Yeast.—The yeast used in these experiments was a strain of *Torulopsis utilis* which had been adapted to growth on ethanol as described by Sperber (5). Samples of approximately 50 gm. of pressed yeast were grown in 8 liter stainless steel Kluver flasks in a medium made up as indicated in Table I. After growth, the suspension was run through a separator and the sedimented yeast washed repeatedly in a centrifuge. Such yeast has a nitrogen content of approximately 7 per cent of the dry weight.

By omitting either the carbon or the nitrogen from the medium it is possible to prepare yeast with nitrogen contents as high as 9 per cent (high N yeast) or as low as 4.5 per cent (low N yeast). Sperber (5) has shown that high N yeast which has grown in excess ammonia contains many buds in various stages of development; active division begins immediately upon addition of a carbon source to the medium. Low N yeast, however, is ripe and contains very few cells in bud. As a result there is a lag period of 4 to 5 hours after addition of ammonia before division begins. Both these yeasts respire actively on endogenous substrate—high N yeast, 3.5 μ l. O_2 consumed/hr./mg. fresh yeast; and low N yeast, 5.5 μ l./hr./mg. (5).

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N¹⁵H₄⁺ Turnover in High N Yeast.—High N yeast was used as a control to study nitrogen turnover in cells that were actively respiring but were neither laying down protein nor dividing. Schoenheimer and coworkers have shown (8) that for mammals in nitrogen balance protein is in a "dynamic state," being continuously degraded and resynthesized. We maintained high N yeast in N¹⁵ ammonium sulfate to determine whether this microorganism, when in "nitrogen balance" also existed in a "dynamic state" as concerns nitrogen metabolism.

47 gm. of fresh high N yeast (9.8 per cent N) were suspended in the medium noted in Table I from which the alcohol was omitted. The ammonium sulfate concentration was 0.06 M with 28.5 atom per cent excess of N¹⁵. After aeration in a Kluver flask at 30° for 3.7 hours, the yeast was centrifuged down, washed twice with water, then with alcohol and ether, and dried.

In this and the other experiments described in this paper the dry yeast was fractionated into total protein and nucleic acid purine portions. This was done as fol-

TABLE I
Composition of Medium for Yeast Growth

Tap water, liter.....	2.
KH ₂ PO ₄ , gm.....	4.5
CaCl ₂ , gm.....	0.4
MgCl ₂ , gm.....	0.4
C ₂ H ₅ OH, gm.....	30
(NH ₄) ₂ SO ₄ , gm.....	9

lows: *Fragmentation:* To break the cell walls, the dry yeast was suspended in alcohol and mechanically vibrated with glass beads (50 cycles/sec. with 1 mm. amplitude for 36 hours (9)). The crushed material was washed with ether and dried. *Removal of phospholipids:* The fragmented yeast was boiled for 2.5 hours with an ethanol-ether mixture (3:1). Approximately 200 ml. solvent were used per 5 gm. yeast. This extracted yeast was washed with ether and air-dried. *Removal of acid-soluble nitrogen:* 10 gm. of solvent-extracted yeast were stirred for 1 hour in the cold room with 150 ml. 0.05 M HCl and then centrifuged for 30 minutes. This extraction was repeated 4 times. *Preparation of protein free of nucleotide:* The acid-washed yeast was extracted successively with 200 ml. 0.1 N NaOH, 150 ml. 0.1 N NaOH, and 75 ml. 0.2 per cent Duponol. The clear brown solution was neutralized with concentrated HCl and then 0.2 volume of 30 per cent trichloroacetic acid was added. The heavy suspension was kept at 90° for 10 minutes to hydrolyze and extract the nucleic acid (10). After cooling and centrifuging, the precipitate was resuspended in 250 ml. 5 per cent trichloroacetic acid and again heated to 90° for 10 minutes. A third extraction was carried out without heating. The extracts were combined as the nucleic acid fraction; the residue—the protein fraction—was washed 3 times with alcohol, twice with ether, and dried. *Separation of nucleic acid purines:* Concentrated HCl was added to the trichloroacetic acid extract to make the solution 1 M in HCl, and the purines liberated by refluxing for 1 hour. Then the pH was adjusted to 8–9 with NH₄OH and refluxing continued for 15 minutes to destroy the trichloroacetate. After aerat-

ing to remove CHCl_3 , the hot solution was filtered and chilled. Upon addition of a large excess of concentrated NH_4OH and 1 M AgNO_3 a white, flocculent precipitate of the silver salts of the purines settled out. Guanine and adenine were separated and purified as described by Levene (11), except that the adenine was not precipitated with picrate but was in part precipitated with β -naphthalene sulfonic acid and recrystallized and in part directly sublimed *in vacuo* at 210–220°. Ultraviolet absorption spectra indicated adenine was free of guanine and *viceversa*.

The fractions taken for analysis were converted to nitrogen gas as described by Rittenberg (12) and the N^{15} content determined in a 60° Nier type mass spectrometer. The results of these measurements are summarized in Table II.

TABLE II
 $\text{N}^{15}\text{H}_4^+$ Assimilation by High N Yeast

Fraction analyzed	Atom per cent excess N^{15}
NH_4^+ in medium.....	28.5
Total yeast.....	0.12
Protein.....	0.04
Guanine.....	0.01
Adenine.....	0.01

There was no detectable fixation in the nucleic acid purines, a trace in the protein, and a small amount in the total yeast. Thus one must conclude that in this yeast in which no net assimilation of nitrogen occurs, there may be a small exchange of NH_3 with the "acid-soluble nitrogen," but synthesis of protein and nucleic acid purine takes place to a negligible extent.

$\text{N}^{15}\text{H}_4^+$ Turnover in Low N Yeast.—When ammonia is added to low N yeast there is a lag period during which nitrogen is actively assimilated but no growth takes place. $\text{N}^{15}\text{H}_4^+$ was administered to such yeast in order to trace its distribution in yeast which was synthesizing protein but not dividing.

50 gm. fresh low N yeast (6.3 per cent N) were suspended in 2000 ml. medium containing no carbon but 12.1 mM of $\text{N}^{15}\text{H}_4^+$ (15.6 atom per cent excess N^{15}). After 1 hour at 30° ice was added and the yeast centrifuged off and washed thoroughly with water. During this incubation, the nitrogen content rose to 8.0 per cent. After washing the yeast with cold 0.05 M HCl , purine analyses were carried out by the method of Graff and Maculla (13), and it was found that the content of purine N remained constant at 0.65 per cent of the dry weight. The experiment was repeated with larger amounts of yeast (24.1 gm. dry weight). The medium contained 24 mM of $\text{N}^{15}\text{H}_4^+$ (31.9 atom per cent excess N^{15}), and during 1 hour of incubation the N content of the yeast rose from 6.55 to 7.34 per cent. Again the purine content remained constant at 0.61 per cent of the dry weight. Only traces of NH_3 were left in the medium after incubation.

The yeast was fractionated as described in the foregoing section, and mass spectrometer analyses are summarized in Table III. In these two experiments 95 to 99 per cent of the NH_3 disappeared from the medium. Yet the N^{15} excess in the medium remained essentially constant indicating either the almost total absence of deaminative degradation processes, or a relatively slow rate of egress for NH_3 formed within the yeast cell. Despite the great assimilation of nitrogen there was no detectable increase in nucleic acid purine. In fact, after N assimilation the ratio of purine N to total N was markedly reduced. However, even though there was no net synthesis of polynucleotide purine, the turnover was rapid as indicated by the high N^{15} content.

TABLE III
 $\text{N}^{15}\text{H}_4^+$ Assimilation by Low N Yeast

Fraction analyzed	Atom per cent excess N^{15}	
	I	II
NH_4^+ in medium		
Before incubation.....	15.6	31.9
After incubation.....	—	30.2
Total yeast.....	1.11	—
Protein.....	0.44	0.88
Guanine.....	0.32	0.73
Adenine.....	—	0.36

$\text{N}^{15}\text{H}_4^+$ Turnover in Dividing Yeast.—The preceding section indicated that turnover of nucleic acids was associated with nitrogen assimilation. Some further information on the course of protein synthesis in *Torulopsis* was obtained by using yeast which had been previously grown in $\text{N}^{15}\text{H}_4^+$ and measuring the dilution of N^{15} during subsequent growth in ordinary NH_4^+ .

30 gm. fresh yeast were suspended in 4 liters of medium containing 28 mM of $\text{N}^{15}\text{H}_4^+$ (12.2 atom per cent excess N^{15}). Ethanol and $(\text{N}^{15}\text{H}_4)_2\text{SO}_4$ were added continuously during growth. After 5 hours (total N added was 65 mM), the suspension was run through a separator and the yeast washed thoroughly with water. One-half of the yeast (I) was removed for N^{15} analyses, and the other half resuspended in medium where the N^{15} had been replaced with ordinary N. After 45 minutes, ice was added to stop growth, and the yeast (II) washed and dried. The extent of growth during this period is indicated in Table IV.

In addition to the fractionations indicated in the previous sections of this paper, several amino acids were isolated from the protein fraction after hydrolysis with 20 per cent HCl. Dicarboxylic amino acids were prepared because of their central position in amination and transamination processes (14, 15). Basic amino acids were sought because basic proteins have been reported (1)

to play an important rôle in protein synthesis. The protein hydrolysate was separated into acid, neutral, and basic fractions by the electrolytic method of Sperber (16). Glutamic acid hydrochloride was precipitated from the concentrated acid fraction with strong HCl. Aspartic acid was obtained from the mother liquor as the copper salt. Arginine was precipitated from the basic fraction with flavianic acid. The latter was subsequently removed by electrolysis. The results of mass spectrometer analyses are summarized in Table V.

TABLE IV
Growth of N^{15} Yeast in Ordinary NH_4^+

Fraction	I	II
Dry weight, gm.	5.49	6.58
Total N, percent.	9.20	9.18
Protein N, percent.	5.58	5.50
Nucleic acid purine N, percent.	0.76	0.77

TABLE V
Distribution on N^{15} in Dividing Yeast

Fraction analyzed	Atom per cent excess N^{15}		Dilution <i>per cent</i>
	I	II	
Yeast.	5.62	4.98	11.5
Protein.	5.58	5.33	3.5
Guanine (nucleic acid)	4.53	4.68	-3.3
Glutamic acid.	5.54	4.53	18.3
Aspartic acid.	5.73	4.93	13.9
Arginine.	4.22	4.35	-3.0

The values in Table V would indicate that most of the ordinary nitrogen had been incorporated in the acid-soluble fraction, the turnover in protein having been only a fraction of that expected from the growth of the yeast. The great dilutions in the glutamate and aspartate fractions indicate that these acids play prime rôles in the fixation of ammonia. This agrees with the work of Roine (15) who has shown that ammonia taken up by *Torulopsis* goes first to the glutamic acid and glutamine of the acid-soluble nitrogen.

In contrast to the dilutions of the N^{15} in the acid amino acids, the basic amino acid, arginine, remained essentially unchanged in N^{15} content as did the nucleic acid guanine. Thus the nitrogenous precursors of these molecules must have been preformed during growth in N^{15} and not significantly diluted during the short period of growth in ordinary nitrogen.

It might be pointed out that a comparison of Tables IV and V reveals that the amount of N^{15} in the yeast has remained constant during growth in ordinary N. This confirms the observation on low N yeast that NH_3 does not pass from the yeast cell into the medium.

DISCUSSION

The work of Schoenheimer and coworkers (8) has indicated the existence of an ammonia pool in a dynamic "equilibrium" with protein nitrogen. Thus even structural protein was reported to undergo continuous degradation and resynthesis. From our results, such a dynamic state between cell and medium seems absent from yeast. Once ammonia is incorporated in the yeast cell it is held permanently. Further work is necessary to determine whether this is caused by relatively irreversible NH_3 fixation or by a low permeability of the cell wall for NH_3 within the cell.

The rapid appearance of ammonia N in the dicarboxylic acids indicates the prime rôle these amino acids play in the fixation of ammonia. The transfer of N to other amino acids is a relatively slow process as indicated by the fact that yeast grown for 5 hours in N^{15} had not reached equilibrium with respect to N^{15} distribution and that the N^{15} of arginine was not diluted by subsequent growth in ordinary N. Further work is necessary to elucidate the exact pathways of NH_3 utilization in yeast.

The evidence that nucleic acids play a rôle in protein synthesis has been based upon observations indicating an increased content of ribopolynucleotide in cells which are laying down protein. We have not confirmed these observations, but rather have noted that during the rapid ammonia assimilation in low N yeast the nucleic acid content remains constant and thus the ratio of nucleic acid N to protein N actually decreases. (We have used purine which is not extracted with cold, dilute acid as a measure of nucleic acid content.) When low N yeast is placed in a medium containing ammonia but no carbon source, it rapidly incorporates N and synthesizes new protein. During the initial part of this process there is no increase in polynucleotide purine content but there is a very rapid turnover as indicated by the appearance of N^{15} in the purines. Thus the data are compatible with the concept that turnover of polynucleotide is associated with the processes of ammonia utilization in yeast.

SUMMARY

By using N^{15} as a tracer the assimilation of ammonia by the yeast, *Torulopsis utilis*, has been studied. It has been shown that:

1. There was no measurable incorporation of N in the protein or polynucleotide purine of carbon-starved yeast.
2. When ammonia is added to nitrogen-starved yeast there is a long lag period before division begins during which the yeast rapidly synthesizes pro-

tein, this process being accompanied by a turnover of polynucleotide purine. There was no significant dilution of the $\text{N}^{15}\text{H}_4^+$ of the medium by ordinary NH_4^+ .

3. When yeast containing N^{15} is allowed to divide and grow in ordinary ammonia, the total amount of N^{15} in the yeast remains constant. The dicarboxylic amino acids are most diluted, while arginine and nucleic acid guanine are not diluted at all.

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THE EFFECT OF SODIUM CHLORIDE ON PHAGE FORMATION BY STAPHYLOCOCCI AT ELEVATED TEMPERATURES

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It has been demonstrated previously that staphylococci which have been subjected to rapid growth in an oxygenated medium at 36°C. for 2 hours and then reduced to a resting state by washing, resuspending the cells in Locke's solution, and maintaining them at 5°C. for 2 hours, possess the ability to produce an immediate six- to tenfold rise in phage activity titre when added to a phage-containing solution (1). This observation was regarded as evidence that actively growing cells contained something which on contact with active phage particles was transformed into phage.

Attempts have been made to define conditions under which cellular reproduction would progress normally while phage formation did not occur either because of the destruction of this factor or the inhibition of its synthesis. Experiments carried out several years ago showed that when a mixture of phage and growing susceptible staphylococci in ordinary nutrient broth was kept at 42.3°C. the bacteria grew without inducing the formation of phage. This was interpreted as being due to inhibition of the production of the phage-augmenting substance (2).

More recent investigations have shown that both the "activated" cells produced by the rapid growth of staphylococci under favorable conditions and the phage-augmenting property of these cells could be quickly inactivated by suspending the bacteria in distilled water and exposing them to 44°C. for a period of 15 minutes; at the same time it was observed that 1 M NaCl protected the "activated" cells against thermal destruction and preserved their ability to immediately give rise to an increase in [phage] when brought in contact with active phage (3). This observation suggested the possibility of providing an environment for the phage-bacterium reaction in which 1 M NaCl might be expected to protect the "activated" cells thereby permitting bacterial growth and phage formation to proceed at temperatures normally inhibitive.

The original purpose of the present investigations was not attained, for it was found that 1 M NaCl interferes with the sorption of phage by susceptible, actively growing bacteria, and thus prevents phage formation.

EXPERIMENTAL METHODS

The following terms are used throughout this paper: (a) [phage] = concentration of phage/ml. expressed as activity units; (b) [phage]₀ = initial concentration of

phage/ml. in activity units; (c) $[\text{phage}]_t$ = final concentration of phage/ml. in activity units; (d) $[\text{phage}_p]_0$ = initial concentration of phage/ml. in plaque units; (e) $[\text{phage}_p]_t$ = final concentration of phage/ml. in plaque units; (f) $[\text{bacteria}]_0$ = initial number of staphylococci/ml; (g) $[\text{bacteria}]_t$ = final number of staphylococci/ml.

These terms refer to the K strain of *Staphylococcus aureus* and to the homologous K phage.

The experimental procedures employed were as follows:

1. [Total bacteria] was determined with a Klett-Summerson photoelectric colorimeter which previously had been calibrated with bacterial suspensions of known density.

2. [Viable bacteria] was ascertained by plating proper dilutions of the suspensions in tryptose agar; counts were made after 24 hours incubation at 36°C.

3. Activity titrations for [phage] were carried out by: (a) removing 4.0 ml. aliquots of the experimental samples and chilling immediately in an ice water bath for 30 minutes; (b) adding 1.0 ml. of a phage solution containing 5.0×10^8 activity units/ml. to each of the 4.0 ml. aliquots; (c) chilling the bacterium-phage mixtures for 10 minutes in an ice water bath; (d) preparing dilutions in tryptose phosphate broth for activity assay according to Krueger's method (4).

4. Plaque counts were made by Gratia's method (5). 0.5 ml. of the experimental sample, properly diluted, was mixed with 3.5 ml. of bacterial suspension containing 16.0×10^7 bacteria/ml.; 1.0 ml. of melted agar was then added and the whole suspension was thoroughly mixed. 1.0 ml. of the latter was removed and spread out on the surface of an agar plate. All plates were read after 18 to 24 hours of incubation at 36°C.

5. [Extracellular phage] in a mixture of phage and bacteria was determined by first passing the mixture through a supercel filter (6). This procedure has been shown to remove all the staphylococci and the associated phage without reducing the concentration of phage free in solution (6). Plaque counts were then done on the filtrate.

6. The growth medium for all experiments was tryptose phosphate broth.

EXPERIMENTAL RESULTS

1. *The Effect of 1 M Sodium Chloride upon Bacterial Viability at 42°C.*—To determine the effect of 1 M NaCl upon bacterial viability at 42°C., staphylococci from an 18 hour agar culture were suspended in either plain broth or in 1 M NaCl broth and incubated at this temperature. Samples were removed at the beginning, after 1.5 hours, and again after 3.0 hours of incubation for determination of total cell counts and viable counts.

Table I summarizes the results obtained. It shows that while cellular reproduction does occur, a considerable number of the staphylococci die. Only some 55 per cent of the cells grown in plain broth are still viable after 3.0 hours of incubation at 42°C. The corresponding viable count for staphylococci suspended in 1 M NaCl is approximately 33 per cent of the total count. It is evident that 1 M NaCl does not exert any measurable protective effect on staphylococci growing at 42°C.

2. *The Effect of 1 M Sodium Chloride upon Production of the Phage-Augmenting Substance at 42°C.*—Suspensions containing 1.0×10^8 bacteria/ml. in either plain broth or in 1 M NaCl broth were prepared from an 18 hour culture of staphylococci. These cell suspensions were then "activated" at 42°C. for a period of 3.5 hours according to the procedure described in earlier reports. At the end of this incubation period total bacterial counts were made and the preparations were tested for capacity to increase the activity titre.

Table II shows that those cells which have undergone incubation at 42°C. for 3.5 hours in plain broth or in broth containing 1 M NaCl retain the capacity to

TABLE I

Effect of 1 M NaCl upon Bacterial Viability at 42°C.

Average values for 2 experiments.

Sample	Incubation	[Total bacteria] _t $\times 10^8$	[Viable bacteria] _t $\times 10^8$
	<i>hrs.</i>		
Staphylococci suspended in plain broth	0	1.0	1.0
	1.5	4.3	2.5
	3.0	6.5	3.6
Staphylococci suspended in 1 M NaCl broth	0	1.0	1.0
	1.5	2.9	1.5
	3.0	6.5	2.1

enhance the phage activity titre. As a matter of fact, certain other experiments in the course of these investigations demonstrated that cells suspended in either plain broth or in 1 M NaCl broth and grown at 45°C. are still capable of producing this effect.

3. *The Effect of 1 M Sodium Chloride upon Bacterial Growth and Phage Formation at 42°C.*—To demonstrate the effect of 1 M NaCl upon bacterial growth and phage production at a temperature of 42°C., suspensions containing 1×10^8 bacteria/ml. in either plain tryptose phosphate broth or in 1 M NaCl-tryptose phosphate broth were prepared from an 18 hour agar culture of *Staphylococcus aureus*. To these suspensions the homologous phage was added so as to obtain a $[\text{phage}]_0$ of 1×10^8 units/ml. The mixtures were shaken for a period of 3 to 4 hours in a water bath kept at a temperature of 42°C. and samples were then removed for activity assay and total bacterial counts.

Table III shows that staphylococci mixed with phage are able to grow equally well in the presence or absence of 1 M NaCl, at least as far as we are able to determine after 3 to 4 hours' incubation at this temperature. However, the presence of 1 M NaCl definitely affects the $[\text{phage}]_t$. A loss of approximately 60 per cent of the $[\text{phage}]_0$ occurs in plain broth mixtures, while those containing 1 M NaCl exhibit neither loss nor increase in $[\text{phage}]_t$.

4. *The Effect of 1 M Sodium Chloride on Sorption of Phage by Susceptible Bacteria.*—Since a culture of staphylococci grown at 42°C. contains "activated" cells among the survivors, it might reasonably be predicted that a mixture of phage and bacteria kept at 42°C. would exhibit some production of phage. However, as noted in the previous section, the opposite appears to be true for $[\text{phage}]_t$ is actually lower than $[\text{phage}]_0$.

TABLE II

Effect of 1 M NaCl upon Capacity of Cells Grown at 42°C. to Increase Phage Activity Titre

Average values for 3 experiments. Activity titrations conducted on mixtures of cells and phage kept at 5°C.

Sample	$[\text{Total bacteria}]_0 \times 10^8$	$[\text{Total bacteria}]_t \times 10^8$	Activity titre $[\text{Phage}]_0 \times 10^8$	Activity titre $[\text{Phage}]_t \times 10^8$
Staphylococci "activated" in plain broth at 42°C.....	1.0	10.0	10.0	160.0
Staphylococci "activated" in 1 M NaCl broth at 42°C.....	1.0	8.8	10.0	150.0

TABLE III

Effect of 1 M NaCl upon Bacterial Growth and Phage Formation at 42°C.

Average values of 3 experiments.

Sample	Incubation	$[\text{Total bacteria}]_0 \times 10^8$	$[\text{Total bacteria}]_t \times 10^8$	Activity titre $[\text{Phage}]_0 \times 10^8$	Activity titre $[\text{Phage}]_t \times 10^8$
	hrs.				
Bacterium-phage mixture in plain broth	3-4	1.0	5.6	1.0	0.39
Bacterium-phage mixture in 1 M NaCl broth	3-4	1.0	5.2	1.0	1.0

One possible explanation of these experimental results is that the considerable number of dead cells accumulating in a staphylococcal suspension grown at 42°C. removes phage from the system through irreversible adsorption, a well recognized property of dead cells. If this is the case, then some change occurs in the presence of 1 M NaCl for the salt mixture shows neither formation nor loss of phage.

In order to get some idea of the differences in adsorptive capacity attributable to 1 M NaCl, suspensions containing 5×10^8 normal, non-activated staphylococci were prepared in either plain broth or in 1 M NaCl broth. The sus-

pensions were then mixed with phage so as to obtain an approximate initial concentration of 1×10^8 plaque units/ml. The mixtures were maintained at 36°C. and samples were removed after 0, 0.5, 1, and 2 hours for determination of [total phage] and [extracellular phage] by the plaque count method.

The results of three experiments have been averaged in Fig. 1. It is clear that the presence of 1 M NaCl entirely upsets the normal process by which phage is adsorbed to the bacterial cell. Very little, if any, phage is taken up; the cells remain uninfected and no significant amount of new phage is formed.

At first glance these results seem to contradict those recorded in section 2, for in the latter case bacteria grown at 42°C. in 1 M NaCl broth give, on the average, a 15-fold increment in phage when tested at 5°C. by the usual procedure. The explanation of this discrepancy may be derived from a separate experiment designed to evaluate the influence of temperature in testing for "activation" of cells.

For this purpose staphylococci were grown in plain broth and in broth containing 1 M NaCl for 2.5 hours at 42°C. One sample was held for 0.5 hour at 5°C.; phage was then added and the mixture was maintained for 10 minutes at 5°C. The cells were then subjected to the usual test for "activation;" in addition, plaque determinations were made on the whole suspension and on supercel filtrates. Another sample of the staphylococcal culture was kept at 42°C., phage was added, and the mixture was held an additional 10 minutes at 42°C. All the measurements mentioned above were made on this sample also. (Table IV.)

The results demonstrate that the temperature imposed in testing the cell suspension for "activation" markedly affects the results. When staphylococci are grown in 1 M NaCl broth at 42°C. for 2.5 hours and are tested at this same temperature for capacity to raise [phage], nearly 85 per cent of the phage added remains extracellular. In contrast, when the test is conducted at 5°C., only 16 per cent of the phage is left free in solution.

Much the same situation is observed in growing mixtures of bacteria and phage maintained at 42°C. When 1 M NaCl is present during the incubation period of 2.5 hours, the final plaque titre is essentially identical with the $[\text{phage}_p]_0$ and 85 per cent of the phage is extracellular. In plain broth without NaCl, the titre is $< [\text{phage}_p]_0$ and only 2 per cent of the phage is extracellular. The plaque determinations cited in Table V incidentally confirm the activity data of Table III.

An important point remains unanswered so far; namely, are the drops in activity titre and plaque count noted in plain broth mixtures of bacteria and phage kept at 42°C. for 2.5 hours due to loss of phage through adsorption on heat-killed staphylococci? If this proves to be the case, one would like to know also why the same or greater loss does not take place in suspensions con-

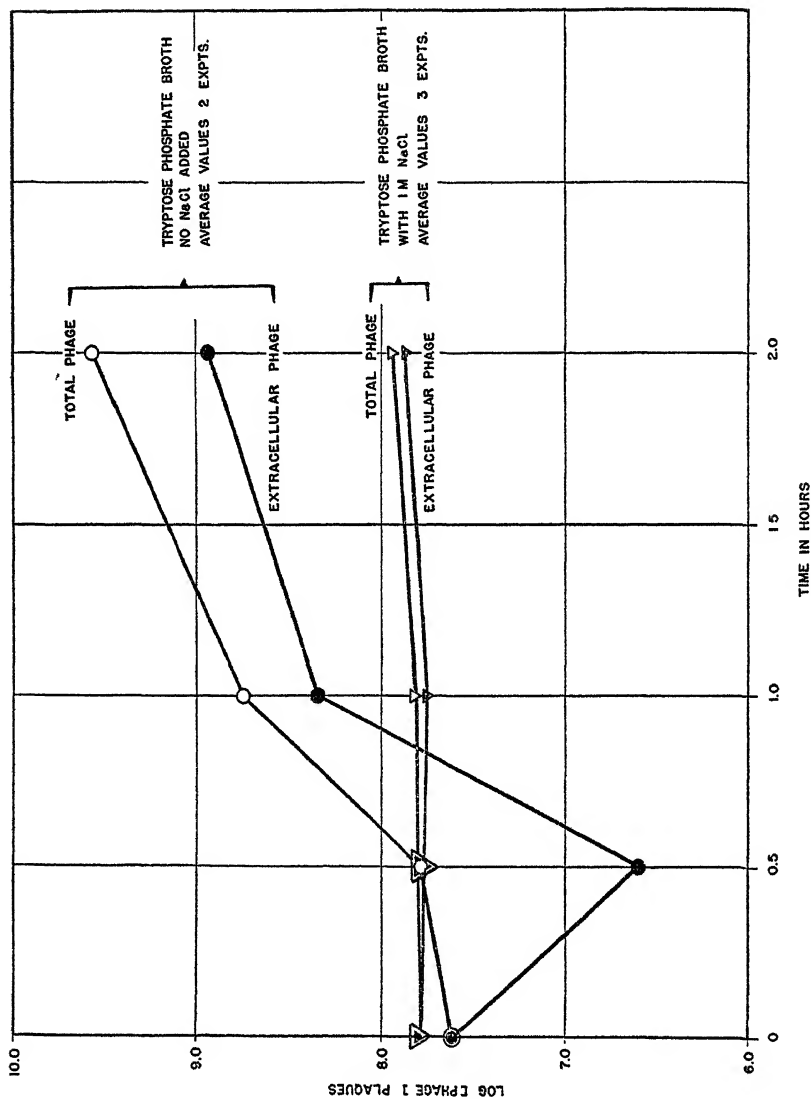


FIG. 1. Curves for [total phage] and [extracellular phage] in mixtures of staphylococci and phage suspended in tryptose phosphate broth and in 1 M NaCl-tryptose phosphate broth at 36°C. 1 M NaCl prevents sorption of phage by bacteria and the cells remain uninfected.

taining 1 M NaCl despite the fact that somewhat more dead cells accumulate here than in plain broth.

Additional experiments were performed to determine how 1 M NaCl affects the sorption of phage by heat-killed staphylococci. Staphylococci grown on nutrient agar were suspended in 1 M NaCl broth and in plain broth. Both

TABLE IV

Effect of Temperature on Results Obtained in Test for "Activation" Conducted with Staphylococci Grown at 42°C.

Sample	Treatment of sample	[Total bacteria] ₀ × 10 ⁸	[Total bacteria] _t × 10 ⁸	[Bacteria] after adding phage × 10 ⁸	Activity titre [Phage] ₀ × 10 ⁸	Activity titre [Phage] _t × 10 ⁸	Plaque titre [Phage] ₀ × 10 ⁸	Plaque titre [phage] _t	
								Total × 10 ⁸	Fil- trate × 10 ⁸
Staphylococci grown 2.5 hrs. at 42°C. in 1 M NaCl broth	Held 5°C. ½ hr. Phage added and kept 10 min. at 5°C.	2.5	10.0	5.0	50.0	700.0	3.3	3.28	0.52
Staphylococci grown 2.5 hrs. at 42°C. in 1 M NaCl broth	Kept at 42°C. Add phage, hold 10 min. at 42°C.	2.5	10.0	5.0	50.0	100.0	3.3	3.4	2.9

TABLE V

Effect of 1 M NaCl on the Distribution of Phage between Staphylococci and Medium at 42°C.

Sample	Plaque titre [phage] ₀ × 10 ⁷	Plaque titre [phage] _t	
		Total × 10 ⁷	Filtrate × 10 ⁷
Staphylococci and phage in 1 M NaCl broth at 42°C. for 2.5 hrs.	6.6	8.2	7.0
Staphylococci and phage in plain tryptose PO ₄ broth at 42°C. for 2.5 hrs.	6.6	2.9	0.06

preparations were kept for 2 hours at 80°C. to kill the organisms. Equal portions of the heat-killed staphylococcal suspensions were mixed with phage and kept either at 5°C. or at 42°C. for 20 minutes. Plaque determinations were carried out on samples removed from the whole suspensions and on filtrates (supernatant).

The results of this experiment (Table VI) may be evaluated by comparing the initial titres with plaque counts at the end of the test period; the drop in plaque count is a measure of the phage taken up by dead bacteria. It is evident that 1 M NaCl vastly alters the adsorptive capacity of heat-killed staphy-

lococci. In the tests conducted at 42°C. for 20 minutes, 65 per cent of the phage in the NaCl mixture is left unadsorbed, while in the absence of NaCl only some 0.3 per cent of phage remains free in solution.

When the experiment is performed at 5°C. the salt effect is somewhat less pronounced. In plain broth 3.5 per cent of the phage is unattached to cocci and when NaCl is present the extracellular fraction is 30 per cent of the total phage added.

Since the growth of staphylococci at 42°C. in tryptose phosphate broth is attended by a considerable accumulation of dead cells in the medium (Table I) and since such cells irreversibly remove homologous phage from solution, an explanation is available for the observed drop in titre (60 per cent loss in Table III and 56 per cent loss in Table V).

TABLE VI

Effect of 1 M NaCl on Sorption of Phage by Heat-Killed Staphylococci at 42°C. and 5°C.

Heat-killed staphylococci	Plaque titre [Phage] ₀ × 10 ⁸	Medium	Temperature	Time	Plaque titre [Phage] _f × 10 ⁸
<i>per ml.</i>			<i>°C.</i>	<i>min.</i>	
5 × 10 ⁸	4.0	1 M NaCl broth	42	20	2.6
5 × 10 ⁸	4.0	1 M NaCl broth	5	20	1.2
5 × 10 ⁸	4.0	Plain broth	42	20	0.011
5 × 10 ⁸	4.0	Plain broth	5	20	0.14

When 1 M NaCl is present in the broth (Table I), even more cells die but they present a less efficient adsorbing surface and most of the phage remains active; *i.e.*, free in solution.

DISCUSSION

Earlier work has shown that the production of phage is inhibited in a broth mixture of staphylococci and phage maintained at approximately 42°C. although the bacteria multiply at a fairly rapid rate (2). More recently Fong (3) has experimented with the thermal destruction of "activated" cells; *i.e.*, staphylococci which have been allowed to grow rapidly in a favorable environment. Normally such organisms when reduced to a resting state at 5°C. possess the ability to produce a six- to tenfold increase in activity titre when added to phage (1). However, Fong found this characteristic to be rapidly abolished by exposure to 44°C. in distilled water along with the capacity of the young cells to survive at this temperature. He noted also that the presence of 1 M NaCl permitted the cells to resist exposure to 44°C. and to retain their phage-augmenting property.

We began the experiments described in the present paper with the idea that the protective function of 1 M NaCl conceivably could be brought to bear on

broth suspensions of staphylococci and phage incubated at 42°C. In this case, we hoped to eliminate the inhibition of phage formation reported by Krueger and Pucheu (2) and ascribed by them to repression of the cellular mechanism responsible for synthesis of phage precursor.

The current experiments were conducted in tryptose phosphate broth and revealed several pertinent facts:

1. Staphylococcal growth at 42°C. in plain broth or in broth containing 1 M NaCl proceeds at a moderate rate. It is attended by measurable rates of death, with the result that at the end of 3 hours in plain broth only 55 per cent of the total cell count represents viable cells; the corresponding figure for the salt-broth preparations is 33 per cent. These results do not appear to be due to the formation of clumps.

2. Cells grown at 42°C. in plain broth or in salt-broth are "activated," *i.e.*, when brought to 5°C. to stop growth and tested with phage they very rapidly raise the activity titre 15- to 16-fold.

3. Mixtures of staphylococci and phage maintained at 42°C. in plain broth exhibit cellular reproduction but fail to increase either the activity titre or plaque count; in fact, the activity titre falls some 60 per cent and the plaque count about 56 per cent. When 1 M NaCl is present the final titre is precisely equal to the initial titre.

4. 1 M NaCl in broth suspensions of staphylococci and phage kept at 42°C. completely alters the distribution of phage throughout the cell population. Normally, phage is taken up very rapidly by the cells but virtually all of it remains extracellular when this concentration of salt is in the medium.

5. A similar result is observed when dead staphylococci are tested for adsorbing capacity. Phage is readily removed from solution by dead cocci and the rate increases with temperature. With 1 M NaCl in the broth, relatively little phage is adsorbed at 5°C. and even less at 42°C.

6. The temperature at which the test for "activation" of organisms grown at 42°C. in NaCl-broth is conducted greatly influences the activity titre obtained. At 42°C. much of the phage remains free in solution; this is reflected in an insignificant rise in phage titre. At 5°C. under the conditions of the test, most of the phage is picked up by the cells with the result that a large increment in activity titre follows.

These experimental data furnish a basis for certain generalizations with reference to the effect of an elevated temperature (42°C.) and increased electrolyte concentration upon the relations obtaining between phage and host cells. In the first place, the increased temperature seems to favor "activation" of staphylococci. The immediate rise in activity titre when phage is added to cells grown at 42°C. and subsequently held at 5°C. to inhibit reproduction, is 15- to 16-fold as contrasted with the 5- to 10-fold increment commonly observed when the growth temperature is 36°C. The addition of 1 M NaCl to the growth medium has no appreciable influence on this phenomenon.

Despite this evidence that staphylococci growing and metabolizing over a period of time at 42°C. efficiently synthesize the substrate required for phage formation, a mixture of phage and cocci maintained at 42°C. not only fails to develop an increase in $[\text{phage}]$ but actually registers a considerable loss. However, if 1 M NaCl is present in the suspension the loss is prevented and $[\text{phage}]_0 = [\text{phage}]_t$. At least two factors appear to participate in this seemingly anomalous result:

(a) If living cells infected at 42°C. behave as they are known to do at 36°C., 20 to 30 phage particles are produced in each bacterium which then undergoes lysis releasing the newly formed phage to infect other organisms. However, the rate of bacterial death at this temperature is considerable and a sufficient number of dead cells accumulates in the medium to provide an adsorbing surface for the uptake of phage. Phage removed from solution in this fashion is irretrievably lost and cannot later participate in the phage-forming reaction which normally ensues when living "activated" cocci and phage are brought into contact. This, then, might be cited as the reason for the observed drop in $[\text{phage}]_t$ in the mixture of phage and bacteria kept at 42°C.

It is possible, also, that the same end result may be attained through the operation of another mechanism. If the bacterium-phage complex were quite thermolabile, $[\text{phage}]$ would be expected to fall off in similar fashion even without the added factor of loss by adsorption on dead cells. Experiments which will be reported in a separate paper indicate that, in fact, such thermal sensitivity on the part of phage attached to susceptible organisms may exist. The data presented here afford no proof that it is an important consideration in the experiments discussed but in certain respects it provides a more logical explanation of the experimental observations than does loss of phage through sorption on dead bacteria. For this mechanism to function there is no necessity to postulate the liberation of newly formed phage under the conditions obtaining in these experiments; even if lysis did not occur, thermal inactivation of phage-cell complexes would produce the observed drop in titre.

If removal of phage through adsorption on dead cells is invoked in the case of the growing mixture, it should operate equally well in the course of testing cocci "activated" by growth at 42°C. in the absence of phage. Viable and total bacterial counts have shown that 45 per cent of the cells present after 3.0 hours are dead. Yet this relatively large fraction somehow fails to compete successfully with the adsorbing surface presented by living "activated" organisms. As a consequence, infection of the "activated" cells occurs and the activity titre exhibits a 15- to 16-fold rise.

It is true that the efficiency of adsorption of phage by dead staphylococci is greater at 42°C. than at 5°C. so that one might be tempted to introduce this difference as a factor in accounting for a positive "activation" test at 5°C. and a negative one at 42°C. However, the degree of difference is minor in com-

parison with the large quantity of phage which could be adsorbed during the periods of exposure of test suspensions to phage.

(b) 1 M NaCl has a pronounced effect on the uptake of phage by living and dead cells. When staphylococci are grown in salt-broth at 42°C. and subsequently are tested for "activation" at 5°C., they adsorb 85 per cent of the phage added and produce a 14-fold increase in activity titre. However, if the test is performed at 42°C. (actually not a satisfactory temperature since it permits continued cellular reproduction) little phage is adsorbed and the increment in activity titre is negligible.

CONCLUSIONS

In experiments with the K strain of *Staphylococcus aureus* and the K race of bacteriophage suspended in tryptose phosphate broth and maintained at 42°C. it was found that the presence of 1 M NaCl produced certain drastic changes in the relationship between the host cells and the infecting virus:

1. Staphylococci grown at 42°C. in plain broth or in NaCl-broth are "activated," i.e. when growth is stopped by lowering the temperature to 5°C. and phage is added, the activity titre immediately displays a rise of 15- to 16-fold.

2. 1 M NaCl tends to prevent the sorption of phage by cocci and this effect is more pronounced at 42°C. than at 5°C. When the activation test is conducted at 5°C. (the usual temperature) most of the phage is picked up by the cells and the described increase in activity titre follows. If the test takes place at 42°C. there is little sorption and correspondingly little rise in phage titre.

3. Mixtures of staphylococci and phage incubated at 42°C. in NaCl-broth fail to produce phage; the final plaque and activity titres are identical with the initial titres. Here, also, the influence of 1 M NaCl in preventing contact of phage with cocci appears to account for the results.

4. Similar mixtures held at 42°C. in plain broth exhibit a drop of about 60 per cent in activity and plaque titres. The loss of phage may be due to adsorption on dead cells accumulating in the suspension or to the thermolability of the bacterium-phage complex, or to both.

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EFFECTS OF HETEROLOGOUS SERA ON FERTILIZED RABBIT OVA

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Blood serum is extensively used in tissue culture and for perfusion of organs. Its nutritive and protective value for the preservation and growth of animal cells *in vitro* is well known. Spermatozoa and ova are cells especially differentiated for fertilization. It has been reported (Chang, 1947) that there is a spermicidal factor in serum which kills spermatozoa of its own species and of other species *in vitro*. This paper deals with a series of experiments which demonstrate a thermolabile factor in the sera of certain species which kills fertilized rabbit ova.

Methods

Blood was collected aseptically by heart puncture with or without anesthesia in the case of rabbits, rats, and guinea pigs. It was obtained from the jugular vein in the domestic animals (cattle, horse, sheep, and goat) except in pigs, when the blood was collected from the neck vessels at slaughter. Fowl blood was obtained by puncturing between cranium and atlas; human blood was drawn from the basilic vein. The whole blood was centrifuged two or three times at 2,000 R.P.M. to secure clear serum. Serum kept at 3°C. no more than 4 days was used in most cases and considered as fresh.

Fertilized rabbit ova were obtained by flushing the excised Fallopian tubes with undiluted rabbit serum 25 to 27 hours after artificial insemination of the does. The does were superovulated by repeated administration of pituitary extracts (Pincus, 1940). Most of the ova thus recovered were at the two-blastomere stage, a few of them were 3-4-blastomere, and a few were fertilized but uncleaved. The ova from four to eight does were mixed up in a watch glass placed inside a Petri dish which contained a piece of moistened filter paper. The ova were transferred to a 10 ml. Carrel culture flask containing 2 to 4 ml. of serum. Manipulation of ova was performed in a glass cabinet at 30°C. Then the flasks were attached to a rocking device (Shapiro, 1939) kept in an oven at 38°C. During operation and manipulation, strict aseptic precautions were observed. After 22 to 25 hours' culture, the ova were examined with a stereoscopic microscope at magnifications of 36 and 72. The numbers of blastomeres in each ovum were recorded.

Ordinarily, when cultured with undiluted rabbit serum, a two-celled ovum would

cleave into 12 to 16 cells, occasionally into 32 cells, after culture for 24 hours. However, some of the ova did not cleave beyond 4-6 cells and a few failed to develop at all. In the treatment of data, all those ova cleaved into 7 cells or more than 7 cells were arbitrarily considered as normally cleaved while those ova without further cleavage or cleaved only to 6 cells were considered dead.

Serum was ordinarily heated at 55°C. for 30 minutes to determine the effect of heating. One serum sample collected from one animal was used. With rats and guinea pigs, the serum was a mixture from several animals unless specially described.

EXPERIMENTAL RESULTS

1. The Recognition of an Ovocidal Factor in the Serum of Certain Species

Data presented in Table I clearly demonstrate that there is an ovocidal factor present in the serum of man, sheep, cattle, goat, and chick. This factor is thermolabile, because there was about 75 per cent of ova cleaved in the serum of man, sheep, cattle, goat, and about 50 per cent in the serum of fowl when these sera were heated at 55°C. for 30 minutes while none cleaved in the normal serum of these animals.

When fertilized rabbit ova were treated with the normal serum of sheep, cattle, and goat for 10 minutes and then cultured in rabbit serum for 24 hours, none of the ova cleaved (Table II). Therefore, the serum of these animals contains a lethal factor rather than a factor which inhibits the growth of rabbit ova. When rabbit ova were treated with normal human serum for 10 minutes, and cultured in rabbit serum, however, a few of the ova cleaved (20 per cent). This indicates that the concentration of this ovocidal factor in the human serum is not so high as compared with the sera of other animals. Of the ova treated for 10 minutes with fowl serum, 14 per cent cleaved; possibly the mode of action of fowl serum differs from that of the ovocidal factor in mammalian sera.

This factor, however, is absent in the serum of rabbit, horse, dog, guinea pig and rat (as shown in Table I), because a large proportion of ova grew in these sera without heat treatment. The data indicate that the rabbit serum is the best for the growth of rabbit ova with or without heat treatment (83 per cent). Horse serum (74 to 80 per cent) is better for the growth of rabbit ova than the serum of guinea pigs (70 per cent) or dogs (55 per cent), with or without heat treatment. Heated pig serum is better for the growth of rabbit ova than is normal pig serum (73 vs. 45 per cent), while normal rat serum is better than the heat-treated serum (75 vs. 38 per cent). It suggests that pig serum may contain a very little ovocidal factor while there may be some nutritive substances in the rat serum which can be destroyed by heat treatment.

Since the serum of rabbit, horse, dog, guinea pig, rat, and pig contains no

TABLE I
Effects of Heterologous Sera on Fertilized Rabbit Ova*

Serum of	Treatment	Condition and No. of ova *								Total	Cleavage
		Dead ova No. of blastomeres				Cleaved ova No. of blastomeres					
		1	2	3-4	5-6	7-8	9-12	13-16			
											<i>per cent</i>
Human	Normal	3	38	10	1	—	—	—	52	0	
	Heated	—	4	4	8	11	27	—	54		70 ± 6.2
Sheep	Normal	7	12	7	—	—	—	—	26	0	
	Heated	—	—	3	11	8	12	11	45		69 ± 6.9
Cattle	Normal	2	26	10	—	—	—	—	38	0	
	Heated	3	2	1	3	15	15	2	41		78 ± 6.5
Goat	Normal	—	8	8	—	—	—	—	16	0	
	Heated	—	1	1	4	6	9	5	26		77 ± 8.3
Fowl	Normal	6	15	14	2	—	—	—	37	0	
	Heated	2	7	8	3	11	7	—	38		47 ± 8.1
Rabbit	Normal	—	1	1	5	7	16	14	44	84 ± 5.5	
	Heated	—	—	2	6	18	14	7	47	83 ± 5.4	
Horse	Normal	—	—	—	9	5	25	6	45	80 ± 5.9	
	Heated	—	—	2	10	10	23	1	46	74 ± 6.4	
Dog	Normal	1	1	5	11	7	10	5	40	55 ± 7.8	
	Heated	—	1	3	14	6	12	5	41	56 ± 7.7	
Guinea pig	Normal	—	2	2	8	17	8	4	41	71 ± 7.1	
	Heated	—	—	—	8	1	12	3	24	67 ± 9.6	
Rat	Normal	—	—	4	5	16	9	2	36	75 ± 7.2	
	Heated	1	4	—	5	—	6	—	16	38 ± 12	
Pig	Normal	—	7	5	11	7	12	—	42	45 ± 7.7	
	Heated	—	2	2	8	10	16	6	44	73 ± 6.7	

* Sera of 3 to 4 animals were tested for each species. Human sera of groups A, AB, B, and O were all tested. Mixed samples and individual samples were tested in the case of rats and guinea pigs.

ovocidal factor, tests were performed to determine whether the combination of any two of these sera would kill the rabbit ova. The results were, however, negative.

2. Further Studies on the Ovocidal Factor

That this ovocidal factor in the serum is unstable is shown in Table III, which illustrates the gradual loss of activity after 17 to 30 days of storage at 3°C.

TABLE II

Cleavage of Rabbit Ova Treated in Heterologous Serum for 10 Minutes and Cultured in Rabbit Serum for 24 Hours

Serum of	Ova dead	Ova cleaved	Total	Cleavage
				<i>per cent</i>
Man	12	3	15	20 ± 10.3
Sheep	7	0	7	0
Cattle	7	0	7	0
Goat	10	0	10	0
Fowl	12	2	14	14 ± 9.3

TABLE III

Instability of Ovocidal Factor of Various Species

Serum of	Age of serum at 2-3°C.	Condition and No. of ova			Cleavage
		Dead	Cleaved	Total	
	<i>days</i>				<i>per cent</i>
Man	17	8	2	10	20 ± 12.6
	22	7	3	10	30 ± 14.5
	30	1	6	7	86 ± 10.9
Sheep	20	7	3	10	30 ± 14.5
	22	6	4	10	40 ± 15.5
	30	4	6	10	60 ± 15.5
Cattle	17	10	0	10	0
	22	5	2	7	28 ± 16.9
	28	6	4	10	40 ± 15.5
	36	2	5	7	71 ± 17.2
Goat	28	7	3	10	30 ± 14.5
	36	5	5	10	50 ± 15.8
Fowl	21	6	4	10	40 ± 15.5
	29	4	3	7	43 ± 18.7

The different strengths of the ovocidal factor in the serum of various species are illustrated in Table IV. When the serum of a particular species was mixed with fresh rabbit serum in various proportions (Table IV), it was found that the percentage of normal cleavage varied. Thus, the concentration or strength

of this factor varies according to the serum of a particular species in ascending order as follows: man, sheep, cattle, goat, fowl.

The data presented in Tables I, III, and IV are, in general, all consistent with the view that the ovocidal factor increases in concentration in the order: man, sheep, cattle, goat (Table IV). The disappearance of this factor in

TABLE IV
Effect of Dilution on Ovocidal Potency

Serum of	Rabbit serum	Condition and No. of ova			Cleavage
		Dead	Cleaved	Total	
	<i>per cent</i>				<i>per cent</i>
Man	25	8	0	8	0
	50	12	10	22	46 \pm 10.6
	75	3	17	20	85 \pm 8
	87.5	3	11	14	79 \pm 10.9
Sheep	25	8	0	8	0
	50	10	4	14	28 \pm 12
	75	3	11	14	79 \pm 10.9
	87.5	2	12	14	86 \pm 9.3
Cattle	25	8	0	8	0
	50	13	0	13	0
	75	1	13	14	93 \pm 6.8
	87.5	1	13	14	93 \pm 6.8
Goat	25	8	0	8	0
	50	13	0	13	0
	75	10	4	14	29 \pm 12.1
	87.5	4	9	13	69 \pm 12.8
Fowl	25	8	0	8	0
	50	10	0	10	0
	75	10	4	14	28 \pm 12
	87.5	5	9	14	64 \pm 12.8

storage is in the same order (Table III). However, fowl serum is more harmful to the rabbit ova (Table IV) and its ovocidal activity is more stable after a longer time of storage (Table III) or of heat treatment (Table I). It may be due to the fact that the fowl is more distantly related to the rabbit evolutionally, so that the serological incompatibility of fowl serum to rabbit ova is more marked. On the other hand, it may be due to the fact that heat treatment removes the ovocidal factor in the fowl serum, but at the same time destroys some nutritive substance in the fowl serum.

The large molecular size of the ovocidal substance was demonstrated by the

following tests: Cattle serum and sheep serum were dialyzed against rabbit serum for 2 to 5 days at 3°C. After culture for 24 hours, rabbit ova cleaved in the rabbit serum but not in the dialyzed cattle or sheep serum. When rabbit ova were cultured in a mixture containing $\frac{3}{4}$ of ultrafiltrate of cattle or sheep serum and $\frac{1}{4}$ of normal rabbit serum, the ova cleaved at a more or less normal rate. It is clear therefore that the ovocidal factor is undialyzable and is of large molecular size.

3. *The Difference between the Ovocidal Factor and the Spermicidal Factor in Serum*

The serum of all the species mentioned in this study, including rabbit and cat, killed rabbit spermatozoa when 0.03 ml. of rabbit semen was added to 1 ml. of the serum. But the serum of certain species (horse, pig, dog, cat, guinea pig, and rat) has no obvious effect on the rabbit ova *in vitro*. Since the spermicidal factor in the serum can be used up by a definite number of spermatozoa (Chang, 1947), tests were performed to determine whether rabbit ova could survive when the spermicidal factor was removed. Rabbit spermatozoa recovered from the vas deferens were added to cattle serum or goat serum till a large proportion of motile spermatozoa were present. The serum was centrifuged and used to culture rabbit ova. It was found that all the ova were dead in the serum unless the serum was inactivated by heat. It is evident, therefore, that the ovocidal factor is not identical with the spermicidal factor.

Since the spermicidal factor in the serum is destroyed or inhibited by Seitz filtration, rabbit ova were cultured in Seitz-filtered cattle and goat serum. It was found that the ova cleaved at a more or less normal rate if the serum was twice filtered. In this respect, the ovocidal factor is similar to the spermicidal factor.

The spermicidal factor can be destroyed or inhibited by sodium citrate (Chang, 1947). Cattle, goat, or rabbit sera containing 0.25 to 1 per cent of sodium citrate were used to culture rabbit ova. It was found that the rabbit ova grew in the rabbit serum but not in the cattle or goat serum. Thus, unlike the spermicidal factor, the ovocidal factor cannot be inhibited or destroyed by the addition of sodium citrate to the serum.

The spermicidal factor has several characteristics similar to the complement (Chang, 1947) which is present in abundance in guinea pig serum. But guinea pig serum has no ill effect on rabbit ova. This is further evidence of the difference between the spermicidal and ovocidal factors.

4. *Other Observations*

Fertilized rabbit ova suspended in saline were subcutaneously injected into rats, guinea pigs, and Dutch rabbits to determine whether these animals can be immunized against rabbit ova. About 20 ova were injected each time into

one animal for 10 weeks at 7 day intervals. Rabbit ova cleaved at a normal rate in the serum of injected rats and rabbits, but not in the serum of guinea pigs (pooled serum or serum from individual animals). This indicates that immunization against rabbit ova can be induced in guinea pigs. But whether it is a specific reaction to ova or to protein in general has not been determined.

Transplantation of rabbit ova (Pincus, 1936; Chang, 1948 *a*) after 24 hours' culture in the serum of other species was carried out to determine whether ova normally cleaved in culture would develop into normal young. One young



FIGS. 1 and 2. Sections of two-celled rabbit ova cultured in normal cattle serum (Fig. 1) or in heated cattle serum (Fig. 2) for 24 hours. Hematoxylin stain. $\times 343$.

was obtained by the transplantation of 18 ova cultured in horse serum into the Fallopian tubes of two recipient does. When 23 ova cultured in heated cattle serum were transplanted into three does, one young was born. Eight young animals were delivered from one recipient mother after transplantation of 14 ova cultured in heated sheep serum into 2 recipient does. No pregnancy occurred by transplantation of 10 ova cultured in heated goat serum.

The results indicate that normal development is possible after the ova are cultured in the heated serum or serum containing no ovocidal factor of other species. Nevertheless, the percentage of normal development (13 per cent) is very low as compared with ova cultured for 24 hours in rabbit serum (28 per cent, Chang, 1948 *b*).

Histological examination of the ova reveals the shrinkage of blastomeres and distortion of nuclear material when the two-celled rabbit ova are cultured

in the serum containing the ovocidal factor (Fig. 1). Although most of the ova cleaved in the heated heterologous sera, the blastomeres were not so regular and their nuclei were eccentric (Fig. 2). This may explain why a very low percentage of ova developed into normal young after cultivation in heterologous sera for 24 hours.

DISCUSSION

Artificial media have long been used for tissue culture. But a medium even as complicated as White's solution (1946) is still better when a certain proportion of serum is added (Jacoby and Darke, 1948). This appears to be due to the proper colloidal osmotic pressure of serum and the presence of a growth factor, amino acids, enzymes, and other unknown constituents in the serum. Blood serum, biochemically and immunologically, is a very complicated system. Whether blood serum may have an ill effect on other tissue cells, as in the case of spermatozoa or ova, is obscure. Parker (1938) reported that a medium containing 15 per cent of serum is better for the growth of chicken monocytes than one with 25 per cent of serum. This may indicate the possibility of adverse effect of serum on other tissues when a harmful factor is introduced in large amount. Seitz filtration of serum, which destroys both spermicidal and ovocidal factors, has been practised in perfusion of organs for aseptic reasons (Carrel and Lindbergh, 1938). This practice may mitigate a possible harmful effect of serum on other tissues, and perhaps for this reason an adverse effect of serum on other tissues has not been stressed.

Carrel and Ebeling (1922) have shown that if serum is inactivated by heat or by shaking, a reduction occurs in the growth activity of homologous cells while heterologous cells show increased activity. They concluded the reduced inhibitory action of treated serum on foreign fibroblasts to be due to the destruction of complement. An increase of inhibiting action of treated serum on homologous fibroblasts is perhaps due to the disappearance of a substance which promotes the activity of the homologous cells. The results in the present study demonstrate, however, that there is no difference in the growth of rabbit ova when heat-treated or normal homologous serum (rabbit) is used. Even in the serum of horse, dog, and guinea pig, which has no obvious harmful effect on rabbit ova, there is no strong evidence to show the destruction of the growth-promoting factor after heating. The growth-promoting factor, therefore, may be not very easily destroyed by the heat treatment used in the present experiments. Heat treatment of heterologous serum (human, sheep, cattle, goat, and fowl) definitely destroyed the toxic effect of foreign serum on rabbit ova; but this toxic substance is not necessarily complement. Only the case of rat serum (Table I) fits the assumption of Carrel and Ebeling that heat treatment inhibits a growth-promoting factor.

The hemolytic reaction of serum to foreign red cells is well known. When a

small amount of washed rabbit red cells is added to the serum of all these species mentioned, a hemolytic reaction occurs. The hemolytic reaction or spermicidal reaction of the serum can be destroyed by heat treatment or by addition of sodium citrate. Since hemolysis requires the participation of complement, the spermicidal factor may be similar to the hemolysin or to one component of the hemolytic reaction. However, the sera of horse, dog, pig, rat, cat, and guinea pig have more or less of a hemolytic reaction on rabbit red cells but have no effect on rabbit ova. This indicates that the ovocidal factor is not due to hemolysin or a component in the hemolytic reaction.

The bactericidal factor in the serum is attributed to the action of complement (Osborn, 1937; Ecker and Lopez-Castro, 1947), and the spermicidal factor in the serum has certain features similar to those of complement. It is possible that the bactericidal factor and the spermicidal factor are the same. As for the ovocidal factor, it is different from complement and from the spermicidal and the bactericidal factor in the serum.

It is very possible that the spermicidal and ovocidal factors in the serum are kinds of enzymes because of their thermolability and large molecular size. It is interesting to note that there is no obvious explanation for the species difference in the presence and concentration of ovocidal factor.

SUMMARY

Undiluted blood serum of various species was used to culture two-celled rabbit ova for 24 hours. It was found that there is an ovocidal factor present in the serum of man, sheep, cattle, goat, and fowl. The factor is lethal rather than inhibitory; exposure to it for 10 minutes will cause the death of the ova. This factor is unstable, thermolabile (destroyed at 55°C. in 30 minutes), and of large molecular size. The strength or concentration of this factor varies according to the origin of the serum, increasing in the order man, sheep, cattle, goat, fowl. The blood serum of rabbit, horse, dog, guinea pig, rat, and pig contains no ovocidal factor against rabbit ova. The ovocidal factor differs from the spermicidal factor, which is present in all the sera of the different species studied with rabbit spermatozoa. Immunization of the guinea pig against rabbit ova is possible. Normal development of young rabbits was obtained by transplantation of ova cultured in the heated or normal serum of other species after 24 hours.

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PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

IV. THE EFFECT OF THE YEAST FRACTION ON VIRUS SYNTHESIS

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It has been previously found (1) that certain non-dialyzable fractions from *S. muscae* and yeast will increase the virus yield of infected cells. In this paper a further analysis of this effect will be reported. It will be shown that in synthetic medium: (a) the fraction greatly increases the virus yield of cells in the log phase, (b) that the active substance is taken up by the cells, and (c) that in the absence of the virus, the cells slowly metabolize the active principle to a form not utilizable for virus reproduction.

RESULTS

From Table I it can be seen that the yeast fraction stimulates the phage yield of infected cells that are in the log phase in synthetic medium. It does not decrease the minimum latent period. The increase in virus formation is fairly proportional to the amount of yeast fraction added up to 2 γ N/ml. (Table II). This confirms the earlier reported observations (1). The yeast fraction has no effect on the multiplication rate of normal cells (Fig. 1).

In veal infusion medium (2) the yeast fraction also stimulates virus reproduction, but to a much smaller degree (Table III). In eight experiments carried out in veal infusion the yeast fraction increased the virus yield in five experiments and had no effect in three experiments.

The Effect of Adding the Yeast Fraction at Various Times.—Table IV shows the effect of adding the yeast fraction at various times to the *S. muscae* system. While the fraction does give a good stimulation of phage reproduction when added to the cells at the same time as the virus, it causes a greater increase in virus formation if incubated with the cells 1 hour before the virus is added.

Further experiments were carried out to determine why the preliminary incubation of the yeast fraction with the cells gave a greater stimulation than adding the fraction and the virus to the bacterial suspension at the same time. Two possibilities were investigated (a) that the bacteria acted on the fairly high molecular weight yeast fraction to liberate a smaller component which was the active substance or (b) that it took some time for the fraction to penetrate into the cells. If (a) was correct, then one would expect to be able to find the active substance in the medium after 1 hour's incubation of the cells with the yeast fraction. If this medium was then centrifuged and new bacteria and

phage added to the medium, a greater increase in virus formation should be expected than would ordinarily be found when the factor and the virus were

TABLE I

The Effect of the Yeast Fraction on Infected Cells in Synthetic Medium in the Log Phase

Two tubes, A and B, each containing 10.0 ml. of synthetic medium plus 10.0 mg. of hydrolyzed casein were inoculated with bacteria from the 18 hour culture to give 2.0×10^7 cells per ml. They were then incubated for $2\frac{1}{2}$ hours. At this time tube A received 0.1 ml. of water and tube B 0.1 ml. of the yeast fraction (111 γ of nitrogen). The tubes were then incubated for 1 hour. The cell count was then 7.3×10^7 cells per ml. 0.1 ml. of virus solution was then added to give a final concentration of 4.7×10^7 virus particles per ml. The tubes were then shaken for 15 minutes and diluted 1:10,000. The final dilution tube of A contained 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein plus 0.1 ml. of water and the final dilution tube of B exactly the same as A, except that 0.1 ml. of yeast fraction (111 γ of nitrogen) was added instead of the 0.1 ml. of water. A one-step growth curve was then carried out on the diluted samples. The table below shows three separate experiments done at weekly intervals.

Experiment	Sample	Minimum latent period	Rise period	Average burst size
		<i>min.</i>	<i>min.</i>	
1	A	30-40	30-40	10
	B	30-40	30-40	50
2	A	30-40	36-46	7
	B	30-40	36-46	20
3	A	30-40	36-46	22
	B	30-40	36-46	46

TABLE II

The Effect of Varying Concentrations of Yeast Fraction on Virus Formation

The experiment was carried out exactly as that described in Table I, except that varying concentrations of yeast fraction were added to the various samples.

Sample	Amount of yeast fraction γ N per ml.	Average burst size
1	—	8
2	0.53	12
3	1.06	20
4	2.12	31
5	4.24	33

added to the cells at the same time. Table V shows that this is not the case. An excess of yeast factor was added to the system in this experiment.

Table VI illustrates that the cells take up some of the substance from the medium and are then able to produce more virus. This is probably the reason

why a preliminary incubation of factor and cells gives a larger virus increase than when the virus and yeast fraction are added at the same time. More of the material has a chance to be taken up by the cells on preliminary incubation and thus the stimulation is greater.

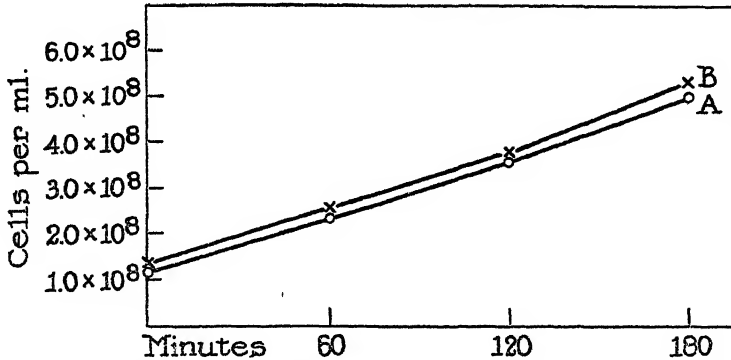


FIG. 1. Two tubes, A and B, containing 10.0 ml. of synthetic medium plus 30.0 mg. of casein were inoculated with 3.4×10^7 cells per ml. from the 18 hour culture and incubated for $2\frac{1}{2}$ hours. At this time tube A received 0.1 ml. H_2O and tube B received 0.1 ml. of yeast fraction (120 γ of nitrogen). This amount of nitrogen gave maximal virus stimulation. The multiplication of cells in each tube was then followed by turbidity measurements.

TABLE III

The Effect of the Yeast Fraction on Virus Formation in Veal Infusion Medium

This experiment was carried out exactly like the experiment described in Table I, except that veal infusion medium was used instead of synthetic medium.

Sample	Average burst size in experiment		
	1	2	3
Control.....	103	92	111
Yeast fraction	123	130	121

This experiment and also the experiment shown in Table V were carried out with an excess of yeast factor so that, although the factor is taken up by the cells, there was enough in the supernatant fluid in the sample in the experiment of Table V to stimulate virus formation.

Cells which have taken up the yeast factor from the medium and so are able to produce more virus lose this property on further incubation in the absence of added phage. This result is shown in Table VII. Penicillin was added in this experiment to prevent complication of the result by growth of the culture. The results show that after a 2 hour incubation period the cells return to their

original virus production. Addition of fresh yeast fraction at this time again increases the yield of virus (*cf.* Experiment 2, Table VII).

TABLE IV

The Effect on Virus Formation of Adding the Yeast Fraction at Various Times

Three tubes, A, B, and C, each containing 10.0 ml. of synthetic medium plus 10.0 mg. of hydrolyzed casein were inoculated with 2.0×10^7 cells per ml. from the 18 hour culture and incubated for 2 hours. At this time tube B received 0.1 ml. of yeast fraction (100 γ of nitrogen). The three tubes were then incubated another hour at which time the cell count was 6.4×10^7 in tube A, 6.7×10^7 in tube B, and 6.7×10^7 in tube C. Tube A then received 0.1 ml. of water and tube C, 0.1 ml. of yeast fraction (100 γ N). 0.1 ml. of virus solution was then added to all tubes to give a final concentration of 4.4×10^7 particles per ml.

The one-step growth curve was then carried out exactly like the one described in Table I. The figures below represent three separate experiments carried out at weekly intervals..

Sample	γ N yeast fraction	Time of preliminary incubation of yeast fraction with cells	Average burst size in experiment		
			1	2	3
A	—	—	10	19	28
B	100	60 min.	40	53	49
C	100	None	25	39	36

TABLE V

The Effect on Virus Formation of the Supernatant Fluid after Preliminary Incubation of the Bacteria with the Yeast Fraction

Three tubes, A, B, and C, were set up containing 10.0 ml. of synthetic medium plus 10.0 mg. of hydrolyzed casein. The tubes were inoculated with cells from the 18 hour culture so as to contain 2.0×10^7 cells per ml. All tubes were incubated for 2 hours. 0.1 ml. of yeast fraction containing 100 γ of nitrogen was added to tube B and all three tubes incubated another hour. The three tubes were then centrifuged for 5 minutes at 5000 R.P.M. and 5.0 ml. of the supernatant fluid from centrifuged tubes A, B, and C were pipetted into separate tubes, A₁, B₁, and C₁. These tubes then received 5.0×10^7 cells per ml. Tube A₁ received 0.1 ml. of water and tube C₁, 0.1 ml. of yeast fraction containing 100 γ of nitrogen. All tubes then received 0.1 ml. of virus solution containing 3.1×10^7 virus particles per ml. A one-step growth curve was then carried out as described in Table I.

Sample	γ N yeast fraction	Time of incubation with yeast fraction	Average burst size in experiment		
			1	2	3
A ₁	—	min.	7	31	37
B ₁	100	60	13	44	58
C ₁	100	0	15	47	54

The Effect of Fractions Prepared from Animal Sources.—Fractions prepared from calf liver, pancreas, and thymus in the same manner as the fractions from yeast and bacteria were not active in stimulating virus synthesis (Table VIII).

Ten times the concentration of the animal fractions used in the experiment shown in Table VIII did not cause any increase in virus reproduction.

The Relationship between the Casein Factor and the Yeast Fraction.—Previous experiments from this laboratory have shown that there is a substance present in hydrolyzed casein which is necessary for the formation of the virus in synthetic medium (3). Various concentrations of hydrolyzed casein were added to the medium together with a constant amount of yeast fraction to see whether the stimulation caused by the yeast fraction would vary with the varying amounts of casein factor. From Table IX it can be seen that the per cent stim-

TABLE VI

The Uptake of the Yeast Fraction by the Bacterial Cells

Two tubes, A and B, containing 10.0 ml. of synthetic medium plus 10 mg. of hydrolyzed casein were inoculated with 8.3×10^7 cells per ml. from the 18 hour culture. Tube A received 0.1 ml. of water and tube B, 0.1 ml. of yeast fraction (120 γ of nitrogen). Both tubes were then incubated for 1 hour. They were then centrifuged at 5000 R.P.M. for 5 minutes and washed in 15.0 ml. of saline and recentrifuged. The centrifuged cells were then each suspended in 5.0 ml. of synthetic medium containing 1 mg. of casein per ml. The cell count was 7.1×10^7 cells per ml. 0.1 ml. of virus solution was added to each sample to give a final concentration of 5.3×10^7 particles per ml. The tubes, A₁ and B₁, were shaken for 15 minutes and diluted 1:10,000 with the final dilution tubes of A and B containing 5.0 ml. of synthetic medium containing 1 mg. of casein per ml. A one-step curve was then carried out. The figures below show the results of three separate experiments.

Sample	γ N yeast fraction in original culture	Average burst size in experiment		
		1	2	3
A ₁	0	7	21	16
B ₁	120	21	39	30

ulation of virus synthesis by the yeast fraction is independent of the amount of casein factor present except at the very low amounts of casein factor where the increase is greater with the yeast fraction than with medium and high concentrations of the casein factor.

A Comparison of the Enzyme Principle with the Virus Fraction.—It had been previously found (1) that the fraction isolated by Reiner and Spiegelman (4), which stimulates the formation of an adaptive enzyme in yeast, can replace the yeast fraction isolated in this laboratory in increasing virus formation. Table X shows that when the fraction of Reiner and Spiegelman gives maximal virus stimulation, the yeast fraction isolated in this laboratory when added to such a system gives no further stimulation. When enough of either fraction is added to give maximal stimulation, the addition of the other fraction does not give a greater virus increase.

TABLE VII

The Effect of Preliminary Incubation Time on the Virus Yield of Cells Which Have Taken Up the Yeast Fraction

Two tubes, A and B, each containing 20.0 ml. of synthetic medium plus 10.0 mg. of hydrolyzed casein were inoculated with 8.1×10^7 cells per ml. from the 18 hour culture and incubated for 2 hours. At this time tube A received 0.1 ml. of water and tube B, 0.1 ml. of the yeast fraction (111% of nitrogen). After 30 minutes both tubes received 0.1 ml. of penicillin (150%). After another 30 minutes, both tubes were centrifuged for 5 minutes at 5000 R.P.M. The cells were washed with 15.0 ml. of saline and centrifuged. These cells were then suspended in 15.0 ml. of synthetic medium containing 1 mg. of hydrolyzed casein per ml. and 10% of penicillin per ml. The cell count was 2.6×10^8 cells per ml. The 15.0 ml. portion of tube A was divided up into three 5 ml. portions, A₁, A₂, and A₃, as was tube B into B₁, B₂, and B₃. 0.1 ml. of virus solution was added to A₁ and B₁ to give a final concentration of 1.1×10^8 virus particles per ml. A one-step growth curve was then carried out on these samples. Tubes A₂ and A₃ and B₂ and B₃ were incubated for another hour at which time tubes A₂ and B₂ were inoculated with virus to give 1.3×10^8 particles per ml. A one-step growth curve was carried out on this sample. Tubes A₃ and B₃ were incubated for another hour and then inoculated with virus to give 1.3×10^8 particles per ml. and a one-step growth curve carried out on this sample. Turbidity measurements showed no cellular multiplication during the 2 hour period of the experiment in Experiment 1.

Experiment 2 was carried out exactly like Experiment 1 except that instead of adding the virus immediately, after 1 hour's incubation and after a 2 hour incubation, the virus was added to A₁ and B₁ immediately and to A₂, B₂, and B₂ + yeast fraction after 90 minutes' incubation. The 0.1 ml. of yeast fraction (95% of nitrogen) was added to a separate sample of B₂ to show that at this time the cells could still respond to the yeast fraction when it was present. It should be noted that penicillin greatly reduces the yield of virus liberated per cell.

Experiment	Sample	Incubation time	Average burst size
		<i>min.</i>	
1	A ₁	0	6
	B ₁		17
	A ₂	60	5
	B ₂		8
	A ₃	120	4
	B ₃		4
2	A ₁	0	5
	B ₁		11
	A ₂	90	3
	B ₂		3
	B ₂ + yeast fraction		8

TABLE VIII

The Effect of Fractions from Various Sources on Virus Formation

The same conditions were used as described in Table I. 10 % of nitrogen per ml. was added to the various samples.

Sample	Additions	Average burst size
1	—	15
2	Yeast	34
3	Thymus	11
4	Liver	18
5	Pancreas	16

TABLE IX

*The Effect of Varying the Concentration of Casein Factor with a Constant Amount of Yeast Factor on Virus Reproduction**

The experiment was carried out as described in Table I except that varying amounts of casein were added to the samples. All even numbered tubes received 10.3 γ of nitrogen of the yeast fraction per ml.

Sample	Casein added per 10.0 ml. <i>mg.</i>	Average burst size	Stimulation <i>per cent</i>
1	0	2	150
2	0 + yeast fraction	5	
3	0.5	5	200
4	0.5 + yeast fraction	15	
5	2.0	16	100
* 6	2.0 + yeast fraction	32	
7	10.0	38	84
8	10.0 + yeast fraction	70	
9	30.0	43	84
10	30.0 + yeast fraction	79	
11	50.0	47	72
12	50.0 + yeast fraction	81	

*One may obtain the result shown below by also using highly purified preparations of the casein factor instead of acid-hydrolyzed casein.

TABLE X

The Effect of the Enzyme-Stimulatory Factor on Phage Formation

The same conditions were used as described in Table I. 11 γ of nitrogen per ml. of the enzyme principle was used and 15 γ of nitrogen per ml. of the yeast fraction isolated in this laboratory was added. Both of these concentrations gave maximal stimulation of virus formation.

Sample	Additions	Average burst size
1	—	14
2	Enzyme principle	38
3	Yeast fraction from this laboratory	34
4	Both fractions	36

DISCUSSION

The results of this paper show that a ribonucleoprotein fraction prepared from yeast stimulates the formation of *S. muscae* virus in cells that are in the log phase in synthetic medium. This fraction has no effect on the multiplication rate of normal cells. It appears, therefore, that this substance plays a rather direct rôle in the synthesis of the virus.

Cells which have been incubated with a solution containing the yeast fraction retain the ability to produce more virus when tested in fresh culture medium, provided the virus inoculation is made soon after the cells are added. After incubation for 2 hours in normal medium in the absence of virus the cells give a normal yield of virus. Since a similar fraction can be isolated from normal *S. muscae* cells, it must be assumed that the cell has a certain concentration of this compound normally present. Furthermore, although the normal cell contains this substance, it must be a limiting one for virus synthesis. When more is added, therefore, it stimulates virus formation. In the absence of the virus, the added amount of substance is slowly metabolized by the cell to some other form not available for virus formation. The concentration of the compound under these latter conditions then returns to normal and no stimulation of virus reproduction is observed.

A phenomenon rather similar to the above experiments has just been reported by Bernheimer and Rodbart (5). Studying the production of streptolysin by *Streptococcus pyogenes*, these authors found that the addition of ribonucleic acid prepared by yeast or from the bacteria themselves increased the formation of the lysin when added to the culture medium. Furthermore, in order to demonstrate the effect, the continued presence of the nucleic acid was required.

A result similar to that with the yeast fraction has been obtained with the casein factor (3). Here again in the absence of the virus the compound is metabolized by the cell to a form not utilizable by the virus for its reproduction. It appears that once the virus infects a cell, it directs synthesis in the cell in such a manner as to form more replicas of itself at the expense of normal cellular synthesis. Cohen (6) has also presented evidence for this theory in his work on the T2r⁺ *E. coli* virus.

The experiments in this paper also lend further evidence to the view that the enzyme-stimulatory principle of Reiner and Spiegelman (4) is similar to the virus-stimulatory fraction isolated in this laboratory. Both fractions can stimulate the synthesis of *S. muscae* virus; furthermore, when a maximum concentration of either principle is added, the addition of the other fraction does not give a further increase in virus reproduction. This observation seems to indicate that both fractions are acting in a very similar manner. Other evidence for the similarity between the two fractions is that neither one is destroyed on incubation with pepsin, trypsin, chymotrypsin, ribonuclease, or desoxyribonuclease (1, 4). Furthermore, both fractions are precipitated maximally at about pH 3.8 under controlled conditions (1, 7). Neither substance is precipitated by ammonium sulfate, trichloroacetic acid, or metaphosphoric acid (1, 7). Both fractions are precipitated by 1 volume of alcohol at an acid pH and also by calcium and alcohol (7). The most highly purified fractions obtained in either laboratory show practically an identical ultraviolet adsorption curve with a maximum at 260 m μ and a minimum at 240 to 245 m μ (1,

7). The final identification of both compounds must of course await the isolation of each substance in pure form. It is tempting to theorize, however, that in the normal cell the substance is used in the synthesis of enzymes and that in the infected cell, in the synthesis of virus. This theory is based on the facts that (a) a fraction very similar to the yeast fraction is found in normal *S. muscae* cells, (b) the fraction stimulates enzyme formation and in infected cells, virus formation, and (c) the normal cell metabolizes the added fraction to a form no longer available for virus synthesis. The recent experiments of Monod and Wollman (8) could also be in accord with this theory. Studying the formation of an adaptive enzyme to lactose in *E. coli*, they found that the infection of the cell by an *E. coli* virus prevented the formation of the adaptive enzyme. This observation could be interpreted to mean that there is a competition between the bacterial enzyme-forming system and the virus for substrates with the virus system predominating. The result of the experiment is not entirely clear, however, since the authors did not show that the enzyme they were studying could form in non-multiplying cells. Since the *E. coli*-infected cell cannot multiply, it is essential that this be done if one is to arrive at their interpretation. Northrop (9) has proposed a scheme for virus formation in which substrates used by the normal cell for the synthesis of enzymes and other cellular proteins would, in the infected cell, be used for the synthesis of the virus. The experiments with this virus system could be taken as evidence for such a theory. To gain a real understanding of the problem with this system, however, it is essential that (a) both the casein factor and the active substance in the yeast fraction be isolated in pure form and then labelled with some radioactive element to follow their metabolic pathway in normal and infected cells, and (b) to study the formation of an adaptive enzyme and a virus in the same host cell under identical and varied conditions. An immunological comparison between the yeast fraction and the bacteriophage may also prove helpful in studying the mechanism of how the yeast fraction increases the formation of *S. muscae* virus.

Methods

Bacterial Cells and Virus.—The cells and virus were determined as described previously (2). Virus solutions were prepared as described previously (3). One-step growth curves of singly infected cells were carried out according to Delbrück and Luria (10). Cells were prepared in the log phase by washing off an 18 hour veal infusion agar slant and inoculating 10.0 ml. of synthetic medium (5), containing 50.0 mg. of hydrolyzed casein with 10^4 cells per ml. and shaking the mixture for 18 hours at 36°C. At this time the cell count was about 2×10^9 cells per ml. Cells from this mixture were then inoculated in 10.0 ml. of fresh synthetic medium containing 10.0 mg. of hydrolyzed casein to give 2.0×10^7 cells per ml. They were then incubated for 3 hours at which time the count was generally around 7.0×10^7 cells per ml.

Preparation of Yeast Fraction.—30 gm. of fresh bakers' yeast was mixed with 30.0 ml. of water in a chilled mortar. Enough powdered glass was added to make a paste and the mixture ground continuously for 20 minutes with a pestle. Then 10.0 ml. of water was added and the mixture ground another 5 minutes. Another 20.0 ml. of water was added and the mixture ground for another 10 minutes. The whole suspension was then centrifuged for 10 minutes at 7000 R.P.M. The supernatant fluid was poured off and recentrifuged for 10 minutes at 7000 R.P.M. The supernatant fluid was then adjusted to pH 3.7 with 10 per cent acetic acid and allowed to stand for 1 hour. The precipitate was centrifuged off and dissolved in 20.0 ml. of water with 0.5 M NaHCO_3 being added to bring the pH to 6.8. The acid precipitation with 10 per cent acetic acid was repeated. The precipitate was suspended in 20.0 ml. of water and adjusted to pH 6.8. This solution was centrifuged. The supernatant fluid was adjusted to pH 4.0 with 10 per cent acetic acid and 1 volume of 95 per cent alcohol added slowly with constant stirring. This mixture was allowed to stand for 1 hour and centrifuged at 5000 R.P.M. for 10 minutes. The precipitate was suspended in 20.0 ml. of water and brought to pH 6.8 with 0.5 M NaHCO_3 and stored in the cold. All operations were carried out at 5°C. The enzyme-stimulatory principle was kindly supplied by Dr. J. Reiner.

*Medium.*¹—The same synthetic medium was used as reported earlier (3). All the hydrolyzed casein was from General Biochemicals, Inc. After the addition of the casein, the medium was brought back to pH 7.4 with N NaOH.

Reaction Mixtures.—All reaction mixtures were shaken at 36°C. as described earlier (2).

I wish to thank Mr. Mortimer Litovchick for excellent technical assistance.

SUMMARY

1. A non-dialyzable fraction from fresh bakers' yeast stimulates the formation of *S. muscae* virus in cells in synthetic medium in the log phase of multiplication.
2. A similar fraction was not found in calf thymus, pancreas, or liver.
3. The active substance in this fraction has been partially purified.
4. This substance is taken up by the cells. In the absence of virus the added substance is metabolized to a form no longer available for virus formation.
5. A purified yeast fraction, which stimulates adaptive enzyme formation in yeast, has been found to stimulate virus formation in the *S. muscae* system.
6. The similarities between the yeast fraction that stimulates adaptive enzyme formation and the yeast fraction that stimulates virus formation are discussed.

¹The strain of *S. muscae* now being used differs from the strain used in earlier work in growing more slowly in synthetic medium. The best conditions for demonstrating the need of the casein factor for virus synthesis with this strain will be published in a subsequent paper.

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TEMPERATURE ACTIVATION OF CERTAIN RESPIRATORY ENZYMES OF STENOTHERMOPHILIC BACTERIA

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I. INTRODUCTION

A review of the literature on thermobiosis reveals many casual observations of the phenomenon, but very few experimental studies which contribute to our knowledge of the mechanism involved (5). In addition to the ability of microorganisms to grow at elevated temperatures, an equally fundamental problem is found in the inability of a group of bacteria, designated as "stenothermal thermophiles" or "obligate thermophiles," to metabolize and reproduce at temperatures which are suitable for most other forms of life. A representative number of these thermophilic bacteria, also used in the present study, were examined earlier and found to exhibit a temperature range for growth of 38–75°C. (4). The effect of a large variety of environmental factors (such as nutrient and nutritive supply, inhibitors, oxygen and carbon dioxide supply, oxidation-reduction potential of the medium, relative hydration of the medium, and pH of the medium) was studied, using the classical methods for measuring growth, as well as a manometric measurement of oxygen consumption. Above 38°C. the stenothermophilic bacteria exhibited very definite requirements with regard to these environmental factors; below 38°C. no combination of conditions could induce these organisms to initiate growth.

References made by several workers to the inactivation of microbial enzymes by low temperatures (3) and to deficiencies, both qualitative and quantitative, in the respiratory mechanism of thermophiles (9, 11) have no supporting experimental data. The absence of growth and proliferation in stenothermophilic cultures below 38°C., however, may be related to the failure of one or more steps in the metastable chain of exothermal and synthetic reactions, so interlinked that the retardation of any single reaction might prevent completely the functioning of others and thus make growth impossible. The temperature range for the respiratory processes, including all chemical processes by which energy is made available to the cell, has been found to be considerably wider than that of growth processes in many organisms. Studies of the activity of bacterial respiratory enzymes as a function of temperature have been limited to organisms with relatively low minimum temperatures for growth.

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In view of the intimate relationship between growth and respiration, this investigation of temperature activation of the respiratory system of a group of bacteria with a very high minimum growth temperature was undertaken with the purpose of (a) determining whether the respiratory system of these thermophiles functions at a temperature below the lower limit of growth, (b) comparing the energies of activation of the complete respiratory system and its enzymic components, and (c) comparing activation energies above and below the critical temperature for growth.

II. EXPERIMENTAL

(a) *Bacterial Cultures*.—Five cultures of stenothermophilic bacteria were selected for study. Obligate thermophilic cultures Nos. 4 and 5 were obtained from the Research Laboratories of the National Canners Association labelled F.S. Nos. 1620 and 4102, respectively. Cultures 1, 2, and 3 were soil isolations. All organisms were facultatively anaerobic, Gram-positive sporulating rods, belonging to the genus *Bacillus*. No taxonomic study was made beyond routine observations necessary to establish the individuality of the cultures in question. These organisms were designated as "stenothermophiles" because of their inability to proliferate at, or below, 37°C.

(b) *Preparation of Bacterial Suspensions*.—The organisms were grown in 1 liter Erlenmeyer flasks, each containing 250 ml. of sterile Bacto-nutrient broth of pH 7.0. Each flask received a 25 ml. inoculum of a 22 to 24 hour nutrient broth culture. The flasks were incubated at 55°C. in a humidified incubator for 22 to 24 hours. Incubation was never permitted to exceed 24 hours. By this procedure the resulting cultures were essentially free of spores. Cells were sedimented by centrifugation at room temperature and washed three times by suspending them in distilled water and centrifuging. The low cell yield per unit of medium made it unsatisfactory to use Sharples supercentrifugation because of the considerable losses involved. Aeration at room temperature to exhaust metabolites was found to be unnecessary. Thorough washing was usually sufficient to eliminate any metabolites adhering to the cells. The organisms collected from 16 flasks and suspended in 6 ml. of M/15 phosphate buffer of pH 7.0 were considered a stock suspension; this was adjusted by dilution in order to obtain the desired activity in the subsequent studies. Storage of suspensions at temperatures from 5–25°C. for 12 hours resulted in loss of activity ranging from 50 to 100 per cent. For this reason all suspensions were used within several hours after preparation.

(c) *Measurement of Dehydrogenase Activity*.—By replacing the cytochrome system by a suitable hydrogen acceptor, methylene blue, which functions also as an indicator, and removing oxygen from the system, the intermediate reactions effected by the dehydrogenases were studied. In Thunberg tubes of 15 ml. capacity were placed 0.5 ml. of 0.05 M substrate, 0.5 ml. of M/15 phosphate buffer of pH 7.0, 0.25 ml. of distilled water, and 0.5 ml. of the resting cell preparation; in the side arm of the stopper, 0.25 ml. of 1:5,000 methylene blue. The tubes, held in an almost horizontal position, were evacuated by means of a water aspirator for a minimum of 5 minutes with constant shaking. Constant temperature air baths were used instead of water baths

to insure temperature equilibration of the material contained in the side arm, as well as in the main chamber. The time required for equilibration was determined directly by observing a thermometer inserted in a control tube. The dehydrogenation was initiated by tipping the methylene blue into the main chamber, and the time required to bring about 70 per cent reduction measured by comparison with an aerobic control consisting of the same volume of substrate, cells, buffer, distilled water, and the appropriate dilution of methylene blue (3:50,000). Recorded values for reduction time were the average of at least triplicate determinations. No effort was made to standardize resting cell suspensions beyond dilution to a reduction time of 4 to 5 minutes at 45°C. for each substrate studied. Visual measurement of reduction through the glass door of the air bath was found to be more satisfactory for this investigation than photocolorimetric measurements. Determinations were carried out at temperatures within the range of 5–55°C. The temperature of the air baths was held constant in the lower ranges to $\pm 0.1^\circ\text{C}$., and in the higher ranges to $\pm 0.2^\circ\text{C}$.

(d) *Measurement of the Activity of the Cytochrome System.*—The cytochrome system, consisting of the very labile cytochromes a and b, stable cytochrome c, cytochrome reductase, and cytochrome oxidase, functions as a chain of electron carriers in transferring hydrogen from metabolites and its final combination with oxygen to form water. Cytochrome oxidase is believed to be the terminal oxidizing enzyme of the respiratory chain of aerobic forms and to effect *in vivo* the oxidation by oxygen of the cytochromes, cytochrome c in particular. To test the activity of this system, a reduced material may be introduced into the system; thus, cytochrome oxidase oxidizes cytochrome c and this in turn oxidized a suitable phenol or amine, hydroquinone or *p*-phenylenediamine. Cytochrome b, on the other hand, in the presence of the oxidase, oxidizes *p*-phenylenediamine but does not attack hydroquinone. By using these substrates, the presence and activity of the various components of the cytochrome system can be determined by manometric measurement of the oxygen consumed by the reactions.

Oxygen consumption was measured by means of Barcroft manometers, calibrated with mercury and checked by the bicarbonate method. In the main vessel of the reaction flask were placed 1 ml. of the appropriate dilution of the resting cell suspension, 1 ml. of *M*/15 phosphate buffer of pH 7.0, and 0.5 ml. of distilled water; in the side arm, 0.5 ml. of *M*/10 substrate (hydroquinone or *p*-phenylenediamine, Eastman grade) in solution of appropriate pH. The control flask contained an equal volume of liquid, but without the bacterial cells and the reduced chemical. Varying degrees of autoxidation of the two chemicals in question made it necessary to run controls in separate manometers and reduce the values of oxygen consumption obtained in the presence of the enzymes by the value of these blanks. Sodium hydroxide solution was not added to the central well of the flasks, since no carbon dioxide was given off. Determinations were made over the temperature range of 20–55°C. for both substrates. The temperature of the insulated and refrigerated water bath was held constant in the higher ranges to $\pm 0.1^\circ\text{C}$., and in the lower ranges to $\pm 0.05^\circ\text{C}$. Temperature equilibration was followed directly in a control flask introduced into the bath at the same time as the test manometer. All gas volumes were corrected for any endogenous metabolism and converted to the standard temperature of 0°C. to make results comparable.

(e) *Measurement of the Activity of Catalase*.—Catalase, an enzyme which disposes of the hydrogen peroxide from a number of biological oxidations, was studied by measuring manometrically the oxygen evolved from a standard solution of hydrogen peroxide by a suspension of washed bacterial cells. In the main vessel of the reaction flask of a Barcroft manometer were placed 1.0 ml. of *m*/15 phosphate buffer, pH 7.0, 0.5 ml. of distilled water, and 1.0 ml. of the resting cell preparation; and in the side arm, 0.5 ml. of 0.3 *M* hydrogen peroxide (prepared from Merck "superoxol"). In the compensating flask, 1.0 ml. of distilled water was substituted for the bacterial suspension. A carbon dioxide absorbent was unnecessary. Equilibration at each temperature over the range from 15–55°C. was followed directly in a control flask immersed in a constant temperature water bath. The reaction was followed by frequent readings of oxygen evolution over a period of several minutes of uninterrupted shaking. All gas volumes were corrected to 0°C.

(f) *Measurement of the Activity of the Complete Respiratory System*.—Aerobic respiration was measured by means of the Barcroft apparatus. Glucose was used as a representative substrate for this study. In the reaction vessel were placed 1.0 ml. of an appropriately diluted resting cell preparation, 1.0 ml. of *m*/15 phosphate buffer of pH 7.0, and 0.5 ml. of distilled water; and in the side arm, 0.5 ml. of *m*/10 glucose (Eastman grade). The compensating vessel contained distilled water in place of the bacterial cells. No carbon dioxide absorbent was employed here because the organisms under investigation did not produce any gas in the fermentation of carbohydrates. By following the usual respirometric procedure, the rate of oxygen uptake was determined over the temperature range from 20–55°C. by correcting the manometer readings for any endogenous metabolism, determined in separate manometers as the oxygen-uptake of the resting cells in the absence of added substrate. All gas volumes were converted to 0°C.

III. RESULTS

(a) *Activity of the Dehydrogenases*.—The log of the rate of reduction of methylene blue was plotted against the reciprocal of the absolute temperature (for organism 3, see Figs. 1 *a* and *b*), and the linear portion of the curve determined. The slope of the line which fitted the plotted points best was found by a statistical analysis of the data for regression coefficients and standard deviation. The energy of activation, μ , was obtained by applying to the data over the linear range the Arrhenius equation:

$$\mu = 4.58 \left(\frac{\log k_2 - \log k_1}{1/T_1 - 1/T_2} \right),$$

where k is the rate, T the absolute temperature, and

$$\left(\frac{\log k_2 - \log k_1}{1/T_1 - 1/T_2} \right)$$

the slope of the curve in the log plot. The activation energies presented in Table I for the five organisms and in Fig. 1 for organism 3 do not include a notation of standard deviation, but are the "best" values to the nearest 500.

For all substrates the rate of dehydrogenation increased exponentially with the temperature up to 45°C., in accordance with the Arrhenius equation. It is considered most significant that at temperatures far below the minimum temperature for growth of these organisms the activation energies for dehydro-

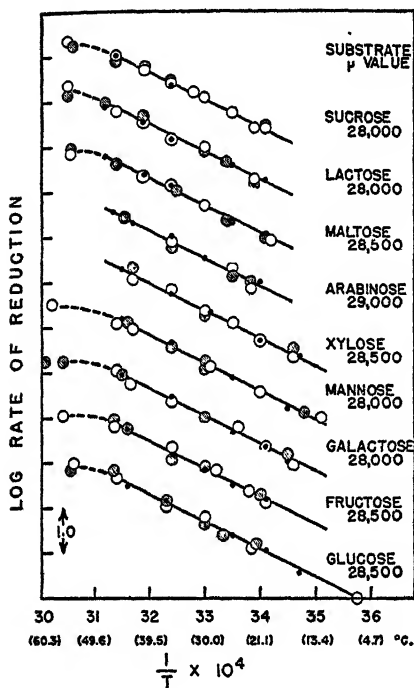


FIG. 1 a

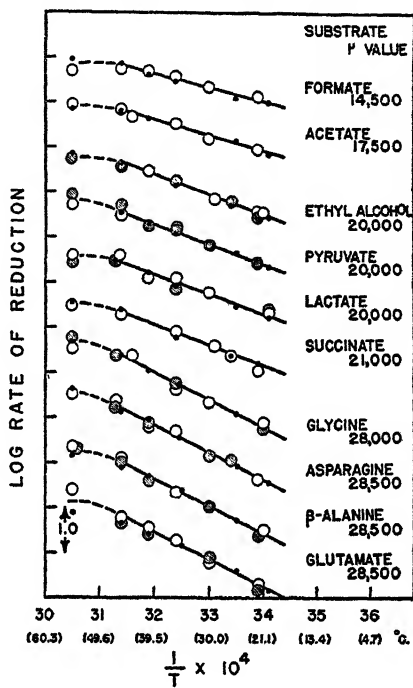


FIG. 1 b

FIG. 1 a and b. Log rate of reduction of methylene blue (to 70 per cent reduction) by resting cell suspensions of organism 3 in the presence of various substrates, plotted against $1/T$.

The three symbols used in designating the points refer to three series of determinations for each substrate made with different bacterial preparations.

genation, are identical with those for temperatures at which the organisms grow rapidly. In all experiments, however, the reaction ceases to follow the Arrhenius equation above 45°C. Although no attempt was made to determine the optimum and maximum temperatures, the data presented in Fig. 1 show that they exceed 45°C.

An analysis of the data obtained for dehydrogenation of the various substrates by washed suspensions of the stenothermophiles, over a temperature range extending far below the minimum temperature for growth, indicates

essentially the same mean activation energy for the following substrates:

28,000 to 28,500 cal. per gm. molecule: glucose, fructose, galactose, mannose, xylose, arabinose, maltose, lactose, sucrose, glycine, β -alanine, monosodium glutamate, asparagine.¹

19,500 to 20,500 cal. per gm. molecule: ethyl alcohol, sodium succinate, sodium pyruvate, sodium lactate, sodium acetate.

TABLE I

Summary of the Dehydrogenase Activity of the Stenothermophilic Bacteria as a Function of Temperature

Substrate 0.0125 M	μ Value					Mean μ value
	Organism No.					
	1	2	3	4	5	
	cal./gm. molecule	cal./gm. molecule	cal./gm. molecule	cal./gm. molecule	cal./gm. molecule	cal./gm. molecule
Glucose (E.K.).*	27,500	29,000	28,500	28,500	28,000	28,300
Fructose (E.K.).	28,500	29,000	28,500	28,000	29,000	28,600
<i>D</i> -Galactose (E.K.).	28,500	27,500	28,000	28,500	28,000	28,100
<i>D</i> -Mannose (E.K.).	28,000	28,500	28,000	29,000	28,000	28,300
<i>L</i> -Xylose (E.K.).	28,000	28,000	28,500	28,000	28,500	28,300
<i>L</i> -Arabinose (E.K.).	27,500	29,000	29,000	29,000	28,500	28,600
Maltose (E.K.).	28,000	28,500	28,500	28,000	29,000	28,400
Lactose (E.K.).	28,000	28,500	28,000	28,500	28,500	28,300
Sucrose (Merck).	28,500	28,000	28,000	28,000	27,000	27,900
Ethyl Alcohol (U.S.P. absolute).	(29,000)	21,000	20,000	20,000	19,500	20,100
Sodium succinate (from E.K. succinic acid).	19,500	20,000	21,000	21,500	20,000	20,400
Sodium pyruvate (from E.K. pyruvic acid).	19,500	19,000	20,000	22,000	21,000	20,300
Sodium lactate (Mallinckrodt 85 per cent reagent lactic acid).	19,000	20,000	20,000	21,000	20,000	20,000
Sodium formate (Baker C.P.).	14,000	15,000	14,500	15,500	17,000	15,200
Sodium acetate (Mallinckrodt U.S.P.).	21,500	19,000	17,500	20,000	20,500	19,700
Glycine (E.K.).	28,500	28,500	28,000	27,500	28,000	28,100
1-Asparagine (E.K.).	28,000	28,500	28,500	30,000	29,500	28,900
β -Alanine (E.K.).	28,000	27,500	28,500	28,500	28,500	28,200
Monosodium glutamate (Amino Product Co.).	28,000	29,500	28,500	28,000	29,000	28,600

* Eastman Kodak Co.

A characteristically different activation energy of 15,000 was obtained for the dehydrogenation of sodium formate.

¹ The temperature characteristic for the dehydrogenation of asparagine has a mean value of approximately 29,000, but the μ values for the individual organisms suggest that this substrate also belongs to the 28,000 to 28,500 category.

(b) *Activity of the Cytochrome System.*—Rates were determined from the slopes of the straight lines in plots of oxygen consumption as a function of time. Typical results are presented in Figs. 2 and 4 for culture 3. By plotting log rate against the reciprocal of the absolute temperature (Figs. 3 and 5), an analysis of the curve for the fit of the points to a straight line indicated the accordance of the data with the Arrhenius equation. The energy of activation

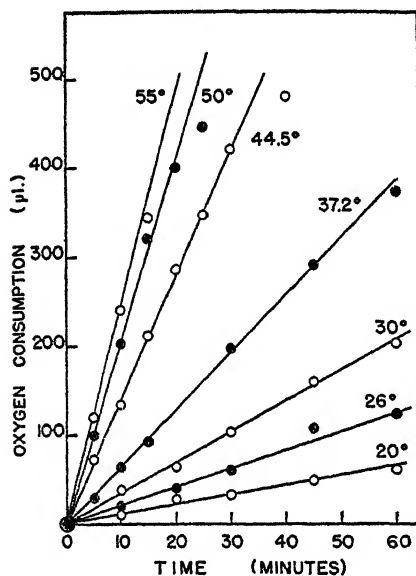


FIG. 2

FIG. 2. Oxygen consumption by resting cell preparation of organism 3 in presence of *p*-phenylenediamine *vs.* time.

$$K_{O_2}^{0^\circ C.} = 2.68$$

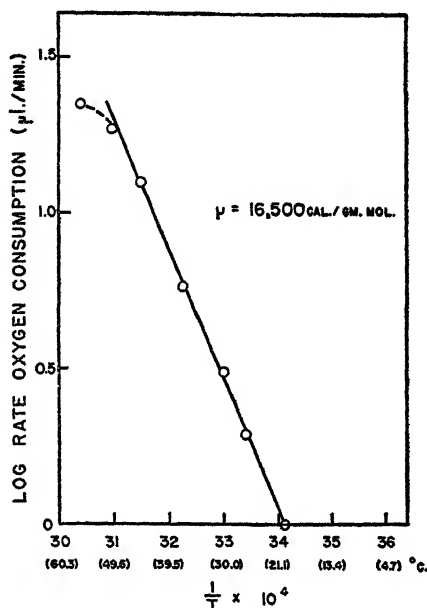


FIG. 3

FIG. 3. Log rate of oxygen consumption by resting cell preparation of organism 3 in presence of *p*-phenylenediamine as a function of $1/T$.

was calculated by substituting the "best" value for the slope of the line in the equation. A summary of results is given in Table II.

The mean activation energy over the temperature range from 20° to at least 45°C. for the cytochrome-*p*-phenylenediamine system, using washed bacterial suspensions of the stenothermophiles was found to be 16,800 cal. per gm. molecule. The mean value for the rate of oxidation of hydroquinone by cytochrome oxidase-cytochrome *c* was somewhat higher, 20,200 calories, and inactivation became apparent at temperatures above 50°C. The data show that the optimum and maximum temperatures for the cytochrome system of these organisms lie above 50°C. The identity of the μ value at temperatures at

which these bacterial cells actively grow and multiply and the μ value at temperatures far below the minimum temperature for growth was noted.

(c) *Activity of Catalase.*—The rate curves are given in Figs. 6 and 7 for dilute and concentrated suspensions of organism 3. The initial straight line portions of the rate curves were used to determine the energy of activation by a plot of

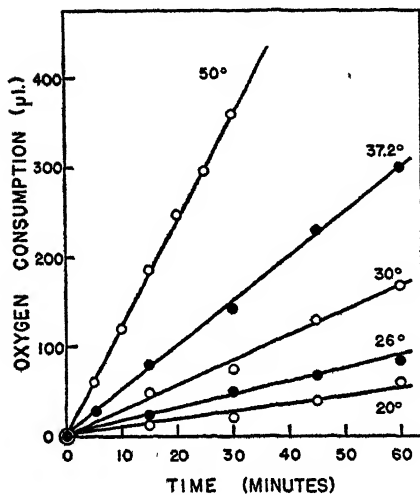


FIG. 4

FIG. 4. Oxygen consumption by resting cell preparation of organism 3 in presence of hydroquinone *vs.* time.

$$K_{O_2}^{0^\circ\text{C.}} = 2.68$$

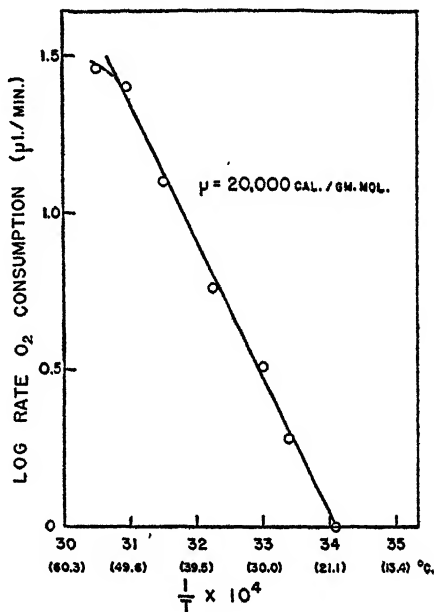


FIG. 5

FIG. 5. Log rate of oxygen consumption by resting cell preparation of organism 3 in presence of hydroquinone as a function of $1/T$.

log rate against the reciprocal of the absolute temperature (see Fig. 8). The μ values calculated from the slopes of these curves for the different cultures are given in Table III.

Below the optimum temperature of the enzyme and far below the minimum temperature for growth of the organisms, the rate of the catalase-catalyzed reaction increases with temperature in accordance with the Arrhenius equation up to about 55°C . The mean μ value for the five stenothermophiles studied was 4,100 calories.

(d) *Activity of the Complete Respiratory System.*—All gas volumes were converted to 0°C ., and oxygen-uptake plotted as a function of time (see Fig. 9).

TABLE II
The Activation Energy of the Cytochrome System of Stenothermophilic Bacteria

Organism No.	Cytochrome oxidase—cytochromes B and C Substrate: <i>p</i> -phenylenediamine		Cytochrome oxidase—cytochrome C Substrate: hydroquinone	
	Range	Activation energy	Range	Activation energy
	°C.	cal./gm. molecule	°C.	cal./gm. molecule
1	22–46	17,100	20–45	19,000
2	22–46	16,600	20–45	20,000
3	20–55	16,500	20–50	20,000
4	20–46	16,800	20–46	19,900
5	20–46	16,800	20–46	22,000
Mean μ value.....		16,800		20,200

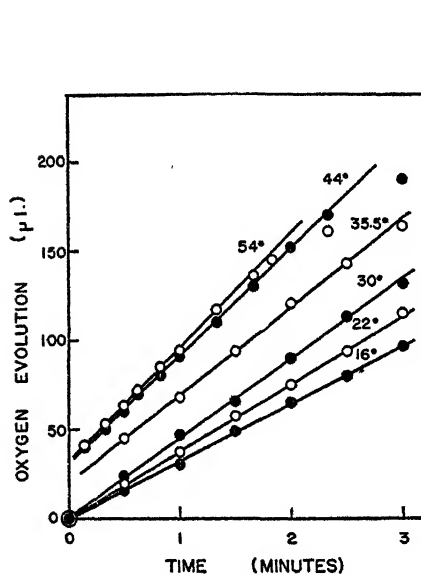


FIG. 6

FIG. 6. Oxygen evolution from 0.05 M H_2O_2 at pH 7.0 by dilute resting cell preparation of organism 3 vs. time.

$$K_{O_2}^{0^\circ C.} = 2.68$$

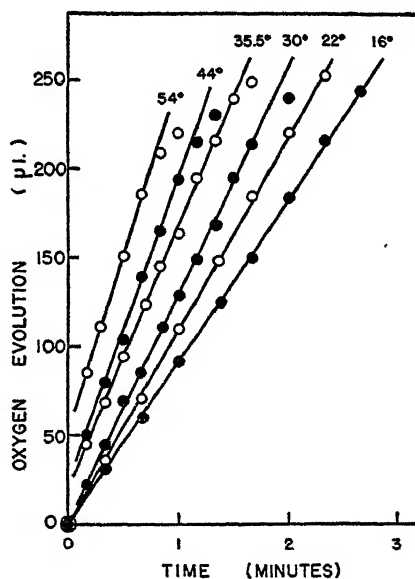


FIG. 7

FIG. 7. Oxygen evolution from 0.05 M H_2O_2 at pH 7.0 by concentrated resting cell suspension of organism 3 vs. time.

$$K_{O_2}^{0^\circ C.} = 2.68$$

When the log of these rates is plotted against the reciprocal of the absolute temperature (see Fig. 10), the data are seen to be in accordance with the Arrhenius equation. Calculated temperature characteristics for the complete

aerobic respiratory system in the presence of added glucose of the stenothermophilic bacteria are given in Table IV.

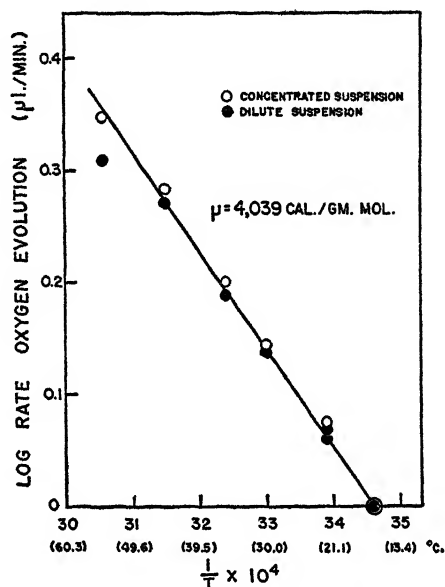


FIG. 8. Log rate of H_2O_2 decomposition by dilute and concentrated resting cell preparations of organism 3 as a function of $1/T$.

TABLE III
The Activation Energy for Catalase of Stenothermophilic Bacteria

Organism No.	Range	Activation energy
	°C.	cal./gm. molecule
1	15-55	4,300
2	20-55	3,900
3	15-55	4,000
4	20-55	4,000
5	15-55	4,200
Mean μ value		4,100

At temperatures below the optimum temperature of the component enzymes of the respiratory system studied earlier, and far below the minimum growth temperature of these organisms, aerobic respiration of the stenothermophilic bacteria, with glucose as a substrate, was found to increase exponentially with temperature in accordance with the Arrhenius equation. The mean activation energy for this group of organisms was 29,500 cal. per gm. molecule.

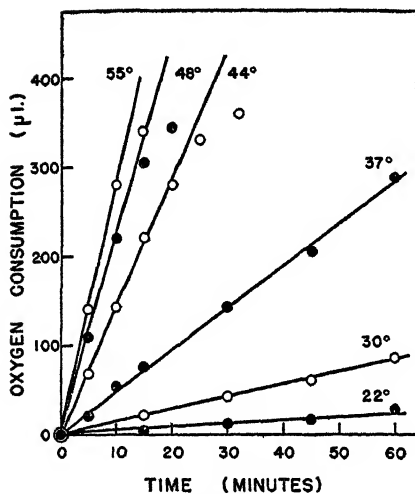


FIG. 9

FIG. 9. Oxygen consumption by resting cell preparation of organism 3 in the presence of glucose vs. time.

$$K_{O_2}^{0^\circ C.} = 2.68$$

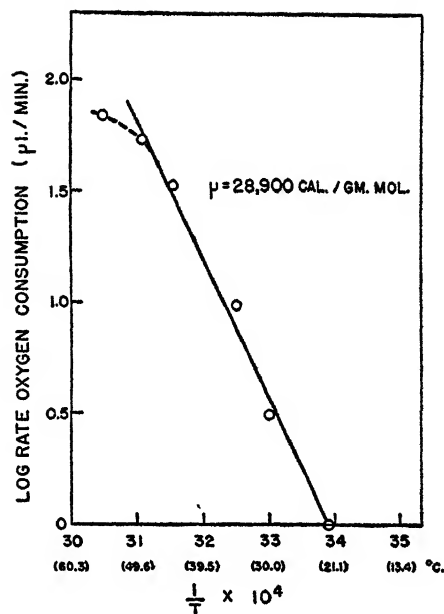


FIG. 10

FIG. 10. Log rate of oxygen consumption of resting cell preparation of organism 3 in the presence of glucose as a function of $1/T$.

TABLE IV

The Activation Energy of the Complete Respiratory System of Stenothermophilic Bacteria

Organism No.	Range	Activation energy
	$^{\circ}C.$	<i>cal./gm. molecule</i>
1	20-50	28,500
2	20-48	29,500
3	22-55	28,900
4	22-55	29,800
5	22-55	30,600
Mean μ value		29,500

IV. DISCUSSION

The energies of activation for dehydrogenation of most substrates are considerably higher than values recorded in the literature, but apparently not high

enough to prevent dehydrogenation reactions in the lower temperature range. The values of 19,500 to 20,500 for the dehydrogenation of ethyl alcohol, sodium succinate, sodium pyruvate, sodium lactate, and sodium acetate compare favorably with the μ value of 19,400 cal. per gm. molecule reported by Gould and Sizer (6) for the dehydrogenation of several of these substrates by *Escherichia coli*. Crozier (2), however, calculated a lower value, 16,700, from Quastel and Whetham's data (15) for the bacterial dehydrogenation of succinate. A distinctly different temperature characteristic of 15,000 for sodium formate was also obtained by Gould and Sizer (6); the stenothermophilic bacteria gave a mean value of 15,000. Carbohydrates (aldopentoses, aldose and ketohexoses, and disaccharides) which yielded μ values between 28,000 and 29,000 in the present study of stenothermophiles, gave μ values ranging from 10,000 to 12,600 for *Rhizobium trifolii* (20), and from 19,400 to 25,000 for *Escherichia coli* (6). An activation energy of 25,000 has also been calculated by Crozier for the dehydrogenation of luciferin by *Cypridina* luciferase (2).

An attempt was made to group the activation energies on the basis of different activating mechanisms, as reviewed by Sizer (17). In a variety of instances the same energy of activation has been found for an enzyme acting on several different substrates. A definite relationship of activation energy to the nature of the substrate has been demonstrated by Sizer (16) for the hydrolysis of sucrose and raffinose by invertase and by Gould and Sizer (6) for the dehydrogenation of acetate, glycine, glutamate, lactate, succinate, glucose, mannitol, galactose, xylose, and sucrose by *Escherichia coli*. This identity of μ values obtained for an enzyme acting on different substrates suggests the possibility that this energy of activation characterizes a particular activating mechanism. A relationship of activation energy to the nature of the substrate was apparent in our study. For the stenothermophilic bacteria, the dehydrogenation of formate gave results consistent with the results of Gould and Sizer (6) and Quastel (14); the dehydrogenation mechanism for glucose, galactose, mannose, xylose, sucrose, glycine, and glutamate gave a common μ value, consistent with Gould and Sizer's analysis. However, the substrates characterized by a μ value of 19,500 to 20,500, although rather sharply defined, had no common chemical basis upon which their association in this group could be explained. Tam and Wilson (20), in a similar temperature-activity study of the dehydrogenase system of *Rhizobia*, obtained values for the various substrates which fell into a high, a medium, and a low group, but they did not feel justified in postulating an activating mechanism for each group.

Temperature activation studies of the cytochrome system of bacteria have not been presented in the literature. Resting cell preparations of the stenothermophilic bacteria in the presence of *p*-phenylenediamine, where the dehydrogenases were not involved in the reaction, gave a mean μ value of 16,800

cal. per gm. molecule at temperatures below the growth minimum. Hadidian and Hoagland (7) using heart extract and the same substrate obtained a value of 9,500. In the presence of hydroquinone, where cytochrome oxidase and cytochrome c alone function, a μ of 20,200 was obtained for the stenothermophiles. Corresponding μ values in the region of 20,000, it may be noted, were obtained earlier in the present study for the oxidation of ethyl alcohol, succinate, pyruvate, lactate, and acetate. An analysis of the data indicates that although both cytochrome b and c are present, cytochrome b contributed only a small fraction to the total activity of these mesocatalysts and is apparently more thermostable than generally accepted (19). Thus, the energy of activation of the dehydrogenase system is much higher than that for the cytochrome system, and indicates that the rate of dehydrogenation in the case of these organisms is the limiting rate of the over-all respiratory process (*cf.* reference 8).

The mean activation energy of 4,100 for the catalase-catalyzed reaction, using resting cells of stenothermophilic bacteria, agrees very well with the value of 4,200 reported for beef liver catalase (18), but does not agree with the 1320–1880 cal. per gm. molecule more recently reported by Bonnichsen, Chance, and Theorell (1) for crystalline catalase of horse blood and liver. It has been suggested that the higher values may be attributed to the presence of partly denatured enzyme. However, the magnitude of the values obtained in our present study of resting cells, in which the enzyme was still in the bacterial cells, is most likely not related to the presence of partly inactivated catalase. Data on the catalase of *Escherichia coli*, from an orientation study in the present investigation, gave a μ value in the region of 6,000 cal. per gm. molecule. Catalase apparently also occurs in higher concentration in the thermophiles than in the mesophiles. Peroxidase has not been demonstrated in any of the five strains of stenothermophilic bacilli (4).

As suggested earlier, a temperature-activity study of the complete aerobic respiratory system in the presence of added glucose of the stenothermophiles should give a μ value representative of the slowest reaction in the respiratory chain. Over the temperature range extending from 20°C. to the point at which inactivation became apparent, the rate of oxygen uptake by a resting cell preparation, in the presence of glucose, increased exponentially with temperature. The mean μ value for the five strains studied was 29,500 cal. per gm. molecule. This value is consistent with the value obtained for the dehydrogenation of glucose, 28,500, and suggests that the dehydrogenation reaction is the rate-controlling reaction in the aerobic respiratory system of these bacteria. The reactions involving the cytochrome system, with a μ value of 16,800, will usually proceed at a greater rate than those catalyzed by the dehydrogenases.

The energy of activation of the respiratory system of other organisms is consistently lower than 29,500 cal. per gm. molecule. Crozier (2), from an ex-

haustive analysis of published data, has found the critical thermal increments of respiratory processes in various plants and animals to be of two statistically significant types: $\mu = 11,500$ and $16,100$ (perhaps also $16,700$). Recent work has substantiated the existence of these modes (*cf.* reference 17). These values have also been found in studies of bacterial respiration: *Rhizobium trifolii*, $10,850$ (21); *Bacillus cereus*, $13,000$ – $18,500$ (10). Lineweaver *et al.* (12) found a slightly higher μ value for *Azotobacter*, $\mu = 19,300$.

The older literature does provide some respiratory data which, when analyzed by the Arrhenius equation, give values for the energy of activation approximating the values found in this study of the stenothermophiles. Crozier's calculations (2) based on the data of Krogh and of von Buddenbrock and von Rohr on winter frogs indicated $28,000$ and $29,500$, respectively, in the lower temperature range. Morales (13), however, has demonstrated that the μ value of the over-all respiratory process of the higher plants and animals is not consistent with the μ value for one of its isolated tissues because the limiting process, or "master reaction," is actually the diffusion of oxygen to the respiring tissue. Thus, there appear to be no values in the literature comparable with the mean value obtained for the respiratory process of the stenothermophilic bacteria.

The most significant result of the present study is the identity of the energies of activation of the respiratory system and its enzymic components obtained at temperatures above and below the minimum temperature for growth of the stenothermophilic bacteria. This observation, therefore, indicates that there is no fundamental difference in the effect of temperature on the respiratory systems of stenothermophilic and mesophilic bacteria. In addition, this may suggest a similarity in the nature of the enzymes functioning in the respiratory process of mesophiles and thermophiles.

V. SUMMARY

The results of this study of the effect of temperature on the respiratory mechanism of five stenothermophilic bacteria may be summarized as follows:—

1. The respiratory mechanism and its various components of the stenothermophilic bacteria were found to function at temperatures below the minimum temperature for growth of these organisms. In every case the rates of the individual reactions involved in the respiratory chain increased exponentially with temperature until the temperature at which inactivation became apparent was reached.

2. The mean activation energies, calculated from the "best" value for the slope of the straight lines resulting from a plot of log rate against the reciprocal of the absolute temperature were:

Dehydrogenases: $28,000$ to $28,500$ calories per gram molecule. Glucose, fructose, galactose, mannose, xylose, arabinose, maltose, lactose, sucrose, glycine, β -alanine, monosodium glutamate, (asparagine).

19,500 to 20,500 calories per gram molecule. Ethyl alcohol, succinate, pyruvate, lactate, acetate.

19,500 to 20,500 calories per gram molecule. Ethyl alcohol, succinate, pyruvate, lactate, acetate.

15,000 calories per gram molecule. Formate.

Cytochrome oxidase and cytochrome b and c (substrate: *p*-phenylenediamine): 16,800 calories per gram molecule.

Cytochrome oxidase and cytochrome c (substrate: hydroquinone): 20,200 calories per gram molecule.

Catalase: 4,100 calories per gram molecule.

Complete aerobic respiratory system (plus added glucose): 29,500 calories per gram molecule.

3. The identity of the energies of activation of the respiratory system and its enzymic components at temperatures above and below the minimum temperature for growth of the stenothermophilic bacteria was demonstrated.

4. An attempt has been made to indicate a relationship between the nature of the substrate and the activation energy by grouping substrates on the basis of common μ values obtained for their dehydrogenation by resting cell preparations of stenothermophilic bacteria. The dehydrogenation reactions have been found to be the rate-controlling reactions in the aerobic respiratory system of these bacteria.

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AQUEOUS HUMOR/PLASMA CHLORIDE RATIOS IN RABBITS, DOGS, AND HUMAN BEINGS*

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A reevaluation of the relative concentration of chloride in the aqueous humor and plasma of various animals will be presented here. Of the many determinations of chloride in these body fluids, only the more recent will be considered. Hodgson reported that the aqueous humor/serum chloride ratio of human beings was 1.13 (1). Davson and Weld found a ratio of 1.075 in unanesthetized dogs (2). Both values appear to exceed by a significant amount the value, 1.04, expected on the basis of the Gibbs-Donnan equilibrium. Hodgson pointed out that free energy must be expended (6.4 calories/liter) to maintain the concentration gradient. Davson concluded either that the extra chloride in the aqueous humor, unaccompanied by extra sodium, potassium, or calcium, must be compensated by a deficiency of some other anion, or that there is present an excess of some organic cation (2). He also attempted to eliminate the possibility that the "discrepant" ratio might be due to an artifact in the experimental procedure. He was not able to account for the high ratio, however, on the basis of instillation of cocaine hydrochloride, variations in the chloride content of the blood, or metabolic activity of the lens.

Actually there is evidence of a relative deficiency of phosphate in the aqueous humor (3), but the deficiency is too small to compensate for the excess chloride. In addition to the reported excess of chloride in the aqueous humor, it is known that bicarbonate ion is also in excess and that the concentration of ascorbic acid which, like chloride, exists in body fluids as an anion, is some 10 to 20 times that of the plasma. Thus, if the chloride ratios referred to above are valid one must assume with Davson either a deficiency of some unknown anion which is present in the blood in significantly higher concentration than it is in the aqueous humor, or the presence in the latter fluid of some unknown cation. The amines appear to represent the only organic cations available from the blood in quantities sufficient to compensate for the cation deficiency, but the concentration of non-protein nitrogen of the aqueous humor (4) is also less than that in the blood, so that this source of cations is not a likely possibility.

Accessory evidence to support the implications of the higher chloride ratios is lacking. Also, data have been obtained in this laboratory, in the course of

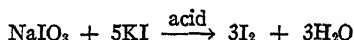
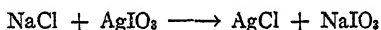
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other experiments, which indicate, on a gram of water basis, that the chloride content of rabbit aqueous humor is less, rather than more, than that of the plasma. For these reasons, comparative analyses by several analytical methods of the chloride concentrations in the aqueous humor and plasma of rabbits, dogs, and human beings have been undertaken.

Methods

Two methods were used for the determination of chloride: the first, that of Schales and Schales (5), a titrimetric procedure based on the reaction $2\text{Cl}^- + \text{Hg}^{++} \rightarrow \text{HgCl}_2$ (not a precipitate). The reaction is carried out in the presence of diphenyl carbazide or diphenyl carbazone, compounds which form violet-blue complex salts in the presence of the mercuric ion. Since HgCl_2 is only slightly dissociated, any mercuric ion which does not combine with chloride is available to form the colored complex and thereby to produce a readily determinable end-point of the titration. The method for plasma analyses can be used without protein precipitation or on Folin-Wu filtrates. Results on the latter are reported to be slightly lower due to absorption of some chloride on the protein. In the present experiments, analyses were carried out both in the presence of protein and with the protein removed.

The second method is that of Sendroy (6), also titrimetric. This method involves the following reactions:



The iodine liberated is determined with $\text{Na}_2\text{S}_2\text{O}_3$ in the usual manner. The Sendroy method also can be employed with or without removal of the protein from the plasma.

Aqueous humor was withdrawn under pontocaine anesthesia, using a micro pipette described previously (7). All samples of aqueous humor from human beings were taken from patients prior to cataract extraction. In these cases both retrobulbar and topical anesthetics were employed. Blood was withdrawn from the antecubital vein of human patients, from the femoral vein of dogs, and by heart puncture from rabbits. In one set of experiments in rabbits, blood was removed and centrifuged under paraffin oil. Approximately 0.20 ml. of aqueous humor, when possible, and 0.20 ml. of plasma, were analyzed. In no instance was less than 0.10 ml. of solution used for analysis.

Heparin was employed as an anticoagulant. The quantity of chloride contributed to the plasma by the heparin solution was determined and found to be negligible compared with the amount present in the plasma.

RESULTS

Duplicate determinations were made of chloride added, in the form of sodium chloride, to aqueous humor of beef eyes, and to plasma of dogs and rabbits. Analyses were made by the methods of both Schales and Sendroy. Protein was not removed from plasma. The results, presented in Table I, indicate that

the average recoveries, by either method, differ by only approximately plus or minus 1 per cent. The concentration of the chloride initially present in the beef aqueous humor was found to be the same, within the experimental error,

TABLE I

The Per Cent Recovery of Chloride Added in the Form of Sodium Chloride to Beef Aqueous Humor and Dog and Rabbit Plasma. Protein Not Removed for Analysis

Beef aqueous humor								
Volume of aqueous humor	Amount of Cl solution added Concentration 500 mm/liter	Calculated Cl		Found Cl		Average recovery	Found Cl	
		Schales	Sendroy	Schales			Sendroy	Average recovery
ml.	ml.	mm/liter	mm/liter	mm/liter		per cent	mm/liter	per cent
1.00	None	—	—	125,	124	100	125, 124	100
2.00	0.30	174	174	174,	172	99.5	172, 173	99
2.00	0.50	200	200	203,	201	101	201, 199	100
1.00	0.50	250	250	250,	250	100	243, 246	98

Dog plasma								
Plasma								
ml.								
1.00	None	—	—	115,	—	100	112, 110	100
1.00	0.15	165	161	167,	166	99	160, 157	98.5
1.00	0.25	192	188	192,	193	100	188, 189	100
1.00	0.50	244	240	240,	246	99.5	237, 238	99

Rabbit plasma								
1.00	None	—	—	101,	102	100	101, 98	100
1.00	1.0	301	300	304,	305	99	292, 299	99
1.00	None	104		104,	104	100		
1.00	0.25	181		182,	183	101		
1.00	0.50	232		232,	233	100		
1.00	0.75	269		267,	269	99.5		
1.00	1.00	297		295,	298	100		
	Water added							
1.00	1.00	52		52,	53	100		
1.00	2.00	34.7		35.3,	35.7	102		

by both methods. Similar results were obtained for rabbit plasma. The concentration of chloride in dog plasma, on the other hand, appeared to be significantly lower by the Sendroy method.

Ascorbic acid dissolved in either aqueous humor or saline solution, in concentrations up to a total of 50 mg. per cent, did not interfere with chloride analyses. Apparently the ascorbic acid was reduced so rapidly by the silver iodate that

it was no longer available to react with sodium iodate produced by reaction of sodium chloride and silver iodate.

Using the Schales method, without deproteination, a preliminary investigation of the ratio of concentration of chloride in the aqueous humor to that in the plasma of six rabbits gave an average value of 0.95. The results of seven additional determinations of the chloride content of the aqueous humor and plasma of rabbits using both analytical methods are shown in Table II. In four

TABLE II
Concentration of Chloride in Aqueous Humor and Plasma of Rabbits

Rabbit No.	Aqueous humor		Plasma			
	O.S.	O.D.	Protein present		Protein removed	
	Schales	Sendroy	Schales	Sendroy	Schales	Sendroy
	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>
1	103.5	105.5	111	108	—	—
2	106*	108*	112	112.5	—	—
3	112	111	112	111.5	—	—
4	109	110*	111.5	109.5	113	113
5	107.5	112.5	107	107*	112.5	113.5
6	112.5	121*	122.5	120.5	123	124
7	114*	115.5	117.5	116.5	117	118.5
Average...	109.2	111.9	113.4	112.2	116.4	117.2

* Single analyses. Others average of duplicate analyses.

Average Ratio of Chloride Concentration Aqueous Humor/Plasma

Method	Protein present	Protein removed†
Schales.....	0.963	0.950
Sendroy.....	0.994	0.980

† Based on last four aqueous values shown in Table I.

of these experiments, the analyses were made on plasma with and without deproteinating. In these experiments most of the samples were arterial blood. The data indicate that the average aqueous humor/plasma chloride ratio is less than 1.0, irrespective of the analytical method used, or whether protein was present or not. The slightly higher ratio with the method of Sendroy is of doubtful significance. With the exception of rabbits 5 and 6 the values for chloride in the aqueous humor by the two methods are in good agreement, despite the small quantity of fluid available for the multiple analyses. The plasma chloride values by the two methods also agree within the probable experimental error. When the analyses were carried out following protein removal, slightly higher values for plasma chloride resulted with both methods.

A third series of experiments was performed on rabbits in which arterial and venous plasma samples were withdrawn from the same animals and their chloride concentrations were compared with those in the aqueous humor. Analyses were performed by the methods of both Schales and Sendroy with protein removed from the plasma samples in all instances. In these experiments, paraffin oil was used to prevent the loss of carbon dioxide before removal of the red cells. The results of the analyses are shown in Table III, where it may be seen that the aqueous humor to plasma ratio of chloride for arterial plasma by both

TABLE III

Concentration of Chloride in Aqueous Humor, and Venous and Arterial Plasma of Rabbits

Aqueous humor		Arterial plasma		Venous plasma	
O.S.	O.D.	Protein removed		Protein removed	
Schales	Sendroy	Schales	Sendroy	Schales	Sendroy
<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>
106.5	109	111	110	105	104.5
108	112	118.5	117	113.5	113
108	107	118	119	112	110
114	115	108	109	111	112
112	109	106	113	106	111
115*	—	118*	—	112*	—
Average. . . . 109.7	110.4	112.3	113.6	109.5	110.1

Average Ratio of Chloride Concentration Aqueous Humor/Plasma

Method	Based on arterial plasma	Based on venous plasma
Schales.	0.984	1.00
Sendroy.	0.975	1.00

* Omitted from average but included in determining ratio of concentrations.

methods was almost identical with the values found previously (Table II); *i.e.*, 0.98. The ratio of concentrations with venous plasma was slightly higher (1.0) by both methods. The table shows that the average values obtained by the two methods for aqueous humor and both arterial and venous plasma were in good agreement.

Table IV (top) shows the results of chloride determinations on the aqueous humor and plasma of dogs. For aqueous humor, the average analytical values for chloride were essentially the same by both the Schales and Sendroy methods. However, it can be seen that consistently higher concentrations of chlorides in the plasma were obtained by the Schales method in every instance, resulting in significantly lower chloride ratios when the samples were analyzed by this method. To check these differences in chloride content of plasma, additional

determinations were made of dog plasma by the two methods, with and without deproteination. Again, in every instance, lower values for chloride were found with the Sendroy method when protein was present (Table IV, bottom).

TABLE IV
Concentration of Chloride in Aqueous Humor and Plasma of Dogs

Aqueous humor		Plasma		Steady state ratio aqueous/plasma	
Schales	Sendroy	Protein present			
		Schales	Sendroy	Schales	Sendroy
<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>	<i>mM Cl/kg. H₂O</i>		
129	133	128	126	1.01	1.05
127	130	130	120	0.98	1.08
123	121	122	113	1.01	1.07
124	118	121	112	1.02	1.05
121	122	123	112	0.98	1.09
121	123	127	117	0.95	1.05
123*	—	122	114	1.01	—
126	127	125	113	1.01	1.13
Average....124.4	124.8	124.7	115.9	0.998	1.078

Plasma				
	Protein present		Protein removed	
	Schales	Sendroy	Schales	Sendroy
Taken from one dog at intervals of about 1 wk.	124	117	121	123
	124	123	125	130
	123	119	125	126
	126	123	124	125
	121	118	121	121
Average.....	124	120	123	125

* Omitted from average but included in determining ratio of concentrations.

The average and individual values (with the exception of the second value, column 6) obtained on dog plasma by the Schaless method with and without protein, and by the Sendroy method without protein, agreed within the experimental errors.

The results of chloride analyses on human aqueous humor and plasma samples are given in Table V. In these experiments all the aqueous humor analyses were performed with the Schaless method only, because the volume of solution was insufficient to permit additional analyses by the Sendroy method. The plasma analyses were performed by both methods, with and without protein.

TABLE V
Concentration of Chloride in Aqueous Humor and Plasma of Human Beings

Aqueous humor	Plasma		Plasma	
Schaes	Schaes Protein removed	Steady state ratio aqueous/plasma	Sendroy Protein removed	Steady state ratio aqueous/plasma
<i>mm Cl/kg. H₂O</i>	<i>mm Cl/kg. H₂O</i>		<i>mm Cl/kg. H₂O</i>	
122	116	1.05	—	—
123	120	1.02	120.5	1.02
122	117	1.04	117.5	1.04
116	114.5	1.01	113.5	1.02
Average.....		1.03		1.03
Protein present		Protein present		
113	116	0.98	—	—
120	119	1.01	115	1.03
122	115	1.06	113	1.09
118.5	117	1.01	110	1.08
Average.....		1.02		1.07

TABLE VI
Summary of Ratios of Concentration of Chloride in the Aqueous Humor to That in the Plasma

Analytical method	Animal	Plasma	Protein present	Protein removed
Schaes	Rabbit	Mixed	0.96 (13)	0.95 (4)
Sendroy	"	"	0.99 (7)	0.98 (4)
Schaes	"	Arterial	—	0.98 (6)
Sendroy	"	"	—	0.98 (5)
Schaes	"	Venous	—	1.00 (6)
Sendroy	"	"	—	1.00 (5)
Schaes	Dog	"	1.00 (8)	—
Sendroy	"	"	1.08 (8)	—
Schaes	Human being	"	1.02 (4)	1.03 (4)
Sendroy	" "	"	1.07 (3)	1.03 (3)

Numbers in parentheses refer to number of experiments.

The table shows that the concentration ratios were slightly more than unity (1.03) when plasma analyses were performed by the Schaes method with and without protein, and by the Sendroy method with protein removed. Higher

ratios were obtained when the Sendroy method was used without removing the protein.

Chloride analyses, using both the Schales and Sendroy method, were made on five additional human plasma samples without removing protein. The values in mm/kg. H_2O obtained for the two methods, in the same order, were 121, 112; 112, 109; 117, 111; 117, 111; and 117, 108. In every instance the Schales method gave a higher value than the Sendroy method. This is consistent with the three values shown in Table V, column 4, and appears to account for the higher values obtained for the concentration ratio by the Sendroy method without removing the protein.

In Table VI the various ratios of concentrations of chloride in the aqueous humor to that in the plasma have been summarized. Reference will be made to these results in the discussion.

DISCUSSION

The ratios of concentration of chloride in the aqueous humor compared with that in the plasma found by the present investigator, for the dog (1.08) and human beings (1.07) by the Sendroy method with protein present, appear to be consistent with those found previously by Davson and Weld (1.075) and Hodgson (1.13). Actually, in the present investigation too few experiments were performed by the Sendroy method on human material with protein present to make more than a qualitative comparison. However, the chloride ratio for human material using the Sendroy method with protein removed, or the Schales method with or without protein, was consistently below that obtained by the present investigator or by Hodgson, both using the Sendroy method with protein present. Similarly, the ratios obtained by the Schales method on the dog (1.00) led to chloride ratios significantly below those obtained by the Sendroy method with protein present. The consistent difference between the results obtained with the Schales method, or with the Sendroy method in the presence of protein, in human and canine material may be ascribed to the lower values obtained by the latter method for chloride in the plasma. No such differences were found with rabbit plasma, where, although a slightly higher ratio was found using the Sendroy method in the presence of protein, essentially identical plasma chloride concentrations were obtained by both methods. The difference in chloride ratios in the rabbit experiments comes not from any differences in plasma chloride concentrations but from slightly different values obtained on the aqueous humor chloride content. The good check obtained (Table IV and V) between the values obtained with protein removed by the Schales and the Sendroy methods on human and canine samples of plasma would suggest that, to obtain accurate values for chloride concentration under the conditions of the experiment with the Sendroy method, the protein should be removed.

The explanation for the lower chloride values obtained by the Sendroy method

in the presence of protein for canine and human plasma, but not rabbit plasma, is not readily apparent. It will be recalled (Table I) that 100 per cent recovery of chloride added to canine plasma was obtained, suggesting that some substances associated with the protein in canine plasma may be reacting with the iodate produced. Sendroy in his original work did not find any differences in chloride content of dog plasma measured by various analytical methods. His experiments do not entirely rule out the possibility that, in the present investigation, sulfhydryl (or other reducing groups on the protein) which might influence the results of the Sendroy method, does not actually account for the discrepant results observed in the presence of protein because, in the present study, both canine and human plasma were not analyzed at once but were stored in the ice box overnight. During this period denaturation may have occurred. The rabbit plasma, on the other hand, was analyzed within an hour after taking samples. Davson and Weld do not state whether they carried out the plasma analyses immediately. However, all the results in the present experiments, with the exception of those obtained by the Sendroy method in dogs and human beings with protein present, indicate that the ratio of chloride in the aqueous humor to that in the plasma does not exceed the value demanded by the Gibbs-Donnan equilibrium. The experimental value, therefore, requires no special explanation,—based, for instance, on postulated excess of some unknown cations or deficiency of other anions in the aqueous humor.

The decrease in chloride ratio found by Davson following paracentesis can be accounted for on other grounds and does not appear to the writer to be inconsistent with the findings cited above. With regard to the higher osmotic pressure found by Hodgson (1), Benham, Duke-Elder, and Hodgson (8) the problem is highly complicated by lack of information concerning relative activities in the aqueous humor or plasma, the reliability of the method in the presence of 8 per cent protein, and other factors, but from the above data it seems unlikely that if an excess osmotic pressure exists, it is due to the chloride ion. It is possible that the ratio of chloride in aqueous humor and plasma is slightly lower for rabbits than for human beings (Table VI). The carbon dioxide ratios appear to vary in the opposite manner; *i.e.*, to be higher in the rabbit than in man.

SUMMARY

The ratio of concentration of chloride in the aqueous humor compared with that in the plasma of rabbits, dogs, and human beings, was determined by the Schales and Sendroy methods. Consistent results were obtained in all the experiments by the Schales method with or without protein, and by the Sendroy method without protein. In the presence of protein, however, lower chloride concentrations were found in the plasma of dogs and human beings by the Sendroy method. The ratios in this instance were higher than values predicted on the basis of Gibbs-Donnan equilibrium and similar to those obtained by

several previous investigators using the same method. With rabbits both methods gave essentially the same results under all conditions.

The ratios (Schales and Sendroy) for the rabbits based on arterial plasma averaged 0.98, on venous plasma 1.00; the ratio (Schales) for the dog for venous plasma was 1.00; the ratio (Schales, protein present or removed; Sendroy, protein removed) for human beings, based on venous plasma, was approximately 1.03. Ratios of 1.08 for the dog and 1.07 for human beings were obtained by the Sendroy method in the presence of protein. Possible explanations for these apparent discrepancies are discussed. In dogs and in human beings, the major evidence supports the contention that the aqueous humor/plasma chloride ratio is not in excess of that predicted by the Gibbs-Donnan equilibrium and therefore requires no special explanation. In the rabbit all the evidence indicates that the chloride ratios are actually less than the value predicted by the Gibbs-Donnan equilibrium.

The analyses in these experiments were carried out by Miss Sylvia Landy.

The aqueous humor and plasma from patients undergoing cataract extraction were kindly provided by Dr. Edwin B. Dunphy and the house officers of the Massachusetts Eye and Ear Infirmary.

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THE ACTIVATION OF UREASE*

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In the course of some heat denaturation experiments on crystalline urease it was noted that certain solutions showed an increase in activity upon moderate heating. This increase upon heating (5 minutes at 60°C.) was shown to be reversible, since upon prolonged cooling the activity decreased and again could be increased by subsequent heating.

Under certain circumstances the phenomenon was found to hold true for crude water extracts of jack bean meal. Furthermore, it was noted that certain solutions of urease showed an increase in activity on standing at room temperature or even on standing in the cold at 2–5°C.

Previous mention of this phenomenon is scarce, although Sumner and Dounce (1) report a moderate increase in urease activity in a solution containing boiled trypsin on standing at room temperature.

In this paper an attempt has been made to explain this phenomenon or, at least, to determine some of the hitherto unrecognized factors which influence the activity of urease solutions.

EXPERIMENTAL

Assay.—Unless otherwise indicated, the activity of the urease solutions was determined in 0.1 ml. portions at 20°C. (10 minutes' temperature equilibration) by adding 1 ml. of a 3 per cent urea solution in 0.67 M phosphate buffer of pH 7.0 (H₂S-treated) and stopping the reaction after 5 minutes with 1 ml. N HCl. In 1 ml. of the reaction mixture the ammonia was determined, according to the method of Conway (2), by diffusion into 1 ml. 0.1 N H₂SO₄. The titration was performed with a semimicro titration setup and electromagnetic stirring. Blank determinations were carried out in the same way, except that hydrochloric acid was added before the enzyme. Such values were negligibly small.

For the determination of the influence of heat on the activity, 0.1 ml. of the urease solution was measured into a small test tube and heated in a water bath at the desired temperature for a definite length of time. After this the tube was placed with the controls in a bath at 20°. After 10 minutes' temperature equilibration the activity measurement was carried out as described above. During the heating and the temperature equilibration the tubes were tightly closed.

In experiments on the influence of freezing, the tubes containing the frozen solution

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were thawed for 5 minutes at 20°C., after which the activity was measured as described, with or without heating.

Experiments with Twice Recrystallized Urease.—Crystalline urease was prepared by the method of Sumner (3) and twice recrystallized according to the method of Dounce (4). An increase in activity was first observed when a solution of this crystalline material dissolved in distilled water was moderately heated. Solutions of the enzyme in phosphate buffer behaved the same way. This increase amounted to as much as 60 per cent when the solution was heated for 5 minutes at 60°C.

Activity determinations in such solutions, however, are apt to be inconsistent unless rigorous precautions are taken to prevent contamination with minute

TABLE I

Relative activity of urease in 0.02 M phosphate buffer at pH 7, showing the influence of H₂S on the change in activity by heating 5 minutes at 60°C. in the presence of 2 per cent gum arabic.

Gum arabic only		Gum arabic plus H ₂ S	
Not heated	Heated	Not heated	Heated
30.0	17.0*	33.4	47.3
32.0	26.8	33.9	47.5
31.9	23.8	34.5	47.3
32.4	13.8	35.1	47.3
Average. . . . 31.6	20.3	34.2	47.4

* No explanation can be given for the irregularity in these determinations.

amounts of metals (5), some of which not only block active sulfhydryl groups, but also catalyze their oxidation by atmospheric oxygen (6). To prevent inactivation of this type Sumner has used as a protecting agent a 2 per cent solution of gum arabic. In our experience, however, solutions in which gum arabic was present did not show an increase in activity on heating; on the contrary, a decrease in activity usually was found on heating for 5 minutes at 60°C. (Table I). Two samples of gum arabic of different origin were tested. It was found, however, that an increase in activity upon heating did occur even when gum arabic was present, if during the heating H₂S was also present (Table I). A similar effect was observed when a ferric salt was added to urease solutions. A solution of urease in 0.02 M phosphate buffer of pH 7, to which ferric chloride had been added in a concentration of 0.001 M showed only a slight increase in activity upon heating, whereas in the same solution a considerable increase was observed if during the heating H₂S was present. The presence of H₂S in a solution containing ferric ion had no influence on the activity at 20°C. without

previous heating (Table II). Since in the samples of gum arabic used traces of iron could be detected, it appears possible that the iron or other metals which may be present are responsible for the negative effect, although this does not

TABLE II

Relative activity of urease in 0.02 M phosphate buffer at pH 7, showing the influence of H_2S on the change in activity by heating 5 minutes at 60°C . in the presence of 0.001 M Fe.

Fe only		Fe plus H_2S	
Not heated	Heated	Not heated	Heated
38.6	42.5	40.3	45.9
40.5	41.7	38.4	49.4
40.6	44.6	40.0	47.3
39.7	—	40.7	49.6
Average . . . 39.9	42.9	39.9	48.0

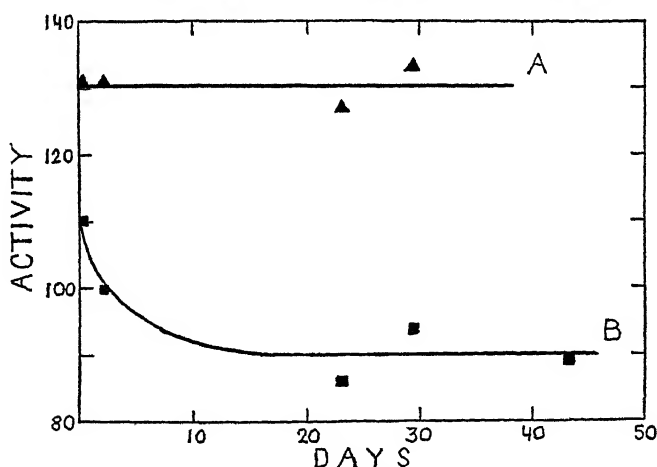


FIG. 1. Activity of twice recrystallized urease in 2 per cent serum albumin, expressed in milligrams of ammonia nitrogen formed by 1 mg. of enzyme in 5 minutes at 20°C . at pH 7.0. Solution kept at $2-5^\circ\text{C}$. Curve A, heated 5 minutes at 60°C . before assay. Curve B, not heated.

explain why gum arabic alone caused a decrease in activity upon heating. For these reasons gum arabic, as a protecting agent, was abandoned in subsequent experiments and a 2 per cent solution of crystalline serum albumin was used instead.

Solutions of urease protected in this way showed an increase in activity on heating for 5 minutes at 60°C ., even in the absence of H_2S . The most remarkable result of the use of serum albumin was, however, that regardless of changes

in the apparent activity of urease solutions on storage in the cold, a constant "potential" activity was found over a period of more than 30 days, provided that the enzyme was heated for 5 minutes at 60°C. before assay. This experiment, illustrated in Fig. 1, was carried out as follows. Twice recrystallized urease (approximately 7.5 mg.) obtained from 100 gm. jack bean meal, was dissolved in 1 ml. water. Immediately thereafter 0.1 ml. of this solution was diluted with 1 ml. of a solution of 2 per cent bovine serum albumin (Armour). This was kept as a stock solution (pH approximately 6). At various intervals 0.1 ml. of the stock solution was diluted once more with 1 ml. 2 per cent serum albumin. One-tenth ml. of this diluted solution was measured into each of 8 tubes, 4 of which were heated for 5 minutes at 60°C. and placed with the unheated tubes in a 20°C. bath. After 10 minutes' temperature equilibration the activities were determined. Points on curve *B* in Fig. 1 represent the activity of the unheated solution and points on curve *A* the activity of the heated samples, each point being an average of at least 3 or 4 determinations which checked within 5 per cent.

The experiment indicated that the activity of the stock solution decreases at first and reaches a constant level after several days standing. The activity of the heated samples on the other hand was approximately the same throughout this period.

Activity determinations carried out on the diluted stock solution after standing in the cold room, showed an increase in activity. For example when the diluted solution made on the 23rd day was preserved in the cold room and assayed 4 days later, the following results were noted:

Activity	
23rd day—	86 units
27th day—	110 units

Likewise for the dilution made on the 30th day an increase in activity was in evidence 6 days later, while the activity of the stock solution diluted on the 49th day was approximately the same as on the 23rd day. Fig. 2 represents a similar experiment with the same preparation of urease crystals as in Fig. 1. All the dilutions were made in the same way, except, that a solution of 2 per cent serum albumin in 0.02 M phosphate buffer was used instead of serum albumin in water. Curves *A* and *B* again represent the activity of the heated and unheated samples as in Fig. 1 and need no further explanation. On the 9th day, however, the dilution of the stock solution was accidentally left at room temperature approximately 1 hour longer than usual. This apparently produced an increase in activity since this value is considerably higher than the other values on that part of curve *B*. Nevertheless on subsequent heating of the solution the same activity as usual was reached. Part of the diluted unheated solution was stored in the cold room and this also resulted in greater activity on standing

(curve *C*). Several of the tubes which on the 9th day had been heated for 5 minutes at 60°C. were placed back in the cold room. The activity decreased again (curve *D*) and approached the same value as the unheated diluted solution standing at the same temperature (note that the dilution of both is the same). Also some of the heated tubes were heated a second time, after 1 and 4 days standing in the cold room. The activity (indicated by points *HH* in the curve) was practically the same in both cases, but lower than the value found after heating the first time.

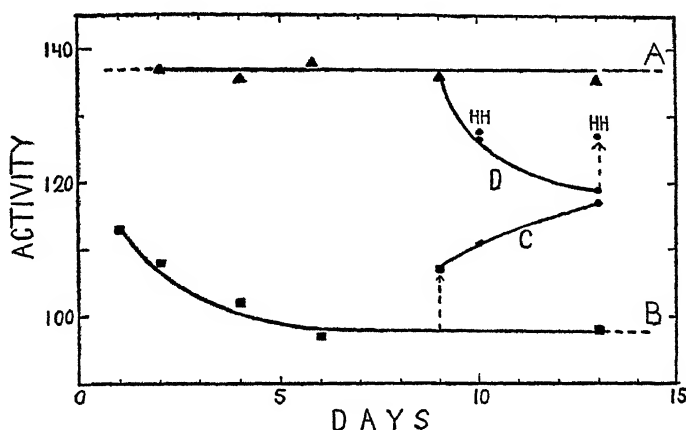


FIG. 2. Activity of twice recrystallized urease in 2 per cent serum albumin in 0.02 *M* phosphate buffer (pH 7), expressed in milligrams of ammonia nitrogen formed by 1 mg. of enzyme in 5 minutes at 20°C. at pH 7.0. Solution kept at 2–5°C. Curve *A*, heated 5 minutes at 60°C. before assay. Curve *B*, not heated. Curve *C*, diluted solution 1:11 standing at 2–5°C. Curve *D*, same as *C*, but after heating 5 minutes at 60°C. For further explanation see text.

This series of experiments shows that the activity is affected by dilution as well as by heating and that the increase in activity upon moderate heating is reversible. It is apparent that the highest activity would be expected in a moderately heated dilute solution. The highest activity reported by Sumner is approximately 133 units per mg. urease. Since none of the solutions tested so far gave a higher activity than 130 to 137 units, even after heating, it could be possible that Sumner's value of 133 units is a "limiting" value. To check this point a fresh preparation of crystalline urease was made. The activity without heating, determined in the same way as in the previous experiments (0.1 ml. of the 1:11 diluted stock solution), was only 83 units per mg., while the same solution diluted fivefold and with 1 ml. heated for 7.5 minutes (instead of 0.1 ml. for 5 minutes, as usual) gave an activity of 151 units per mg.

It can be concluded, therefore, that the activity of crystalline urease deter-

mined under standard conditions with the enzyme as limiting factor and with no irreversible inactivation taking place, is not a function of the amount of enzyme alone. It is influenced by the time of standing, the concentration, and the temperature prior to the determination.

Experiments with a Crude Water Extract of Jack Bean Meal.—All data represented in this section have been obtained with a single crude water extract of jack bean meal. This extract was prepared by stirring 15 gm. of jack bean meal (Arlco) with 150 ml. of water at room temperature for a few minutes, af-

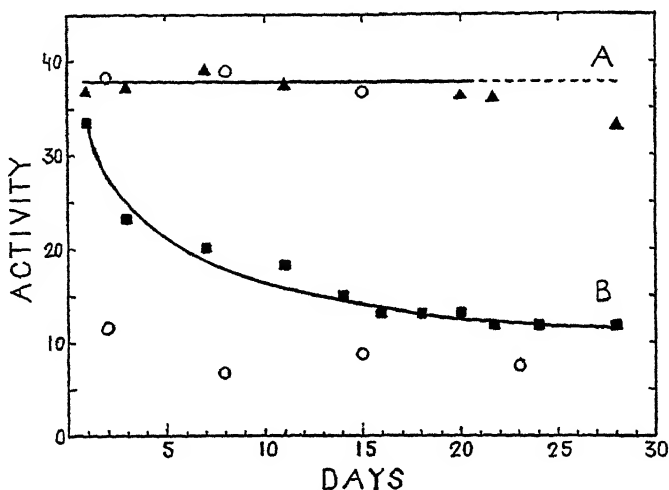


FIG. 3. Relative urease activity of a crude water extract of jack bean meal 1:15, expressed in micromols ammonia formed per milliliter reaction mixture at 20°C. Solution kept at 2–5°C. Influence of time of standing, heating, and freezing. Curve A, activity after heating 5 minutes at 60°C. of the unfrozen solution (triangles) and of the frozen solution after thawing (circles). Curve B, activity during storage at 2–5°C. Circles below this curve represent activity after 24 hours' freezing at –10°C.

ter which the suspension was centrifuged and kept in the cold room at 2–5°C. By the next day a precipitate had settled out and was centrifuged off. The pH of the slightly hazy supernatant (6.2) was brought to 7.0 by the addition of NaOH, which resulted in a clear solution on which the first activity determinations were carried out (first points on curves A and B of Fig. 3). During the following day more precipitate settled out, which was again centrifuged off. This procedure which was repeated a few times, apparently did not interfere with the "potential" activity of the solution, since each time approximately the same activity was found after 5 minutes' heating at 60°C. (curve A in Fig. 3). It did, however, cause an increase in the pH of the solution, which after 15 to 20 days had gone up to approximately 8. By this time the activity of the ex-

tract (on standing in the cold room) had become nearly constant but the activity following heating tended to fall off (Curve *A* in Fig. 3 terminal portion). This could be caused by a lesser heat stability of the enzyme at that higher pH.

Unless otherwise indicated, the experiments with the crude extract were carried out in the same way as in the previous section except that 0.1 ml. portions were used without any dilution for determining the activity. In addition to the influence of moderate heating and of the time of standing, Fig. 3 also shows the influence of freezing and subsequent thawing and heating (activ-

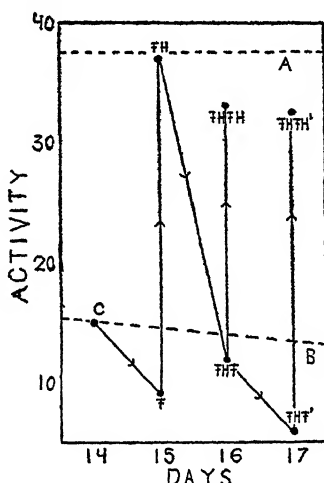


FIG. 4. Relative urease activity of a crude water extract of jack bean meal 1:15, expresses in micromols ammonia formed per milliliter reaction mixture at 20°C. Reversibility of inactivation by freezing. Influence of time of freezing. Curves *A* and *B*. Same as in Fig. 3. *C*, activity of the extract after 14 days' standing at 2–5°C. Solution frozen for 24 hours at –10°C. and thawed (*F*), heated for 5 minutes at 60°C. (*FH*), frozen again for 24 hours (*FHF*), and heated again (*FHFH*). Also, frozen for 48 hours (*FHF'*) and subsequent thawing and heating (*FHFH'*).

ity indicated by open circles). After various periods of storage in the cold room, 2 samples of 0.1 ml. of the extract were frozen at approximately –10°C. After 24 hours in the frozen state the samples were thawed and the activity determined with and without previous heating. Freezing caused an additional inactivation as compared with the activity of the extract in the cold room on that same day. This additional inactivation was smaller, the more the extract had been already inactivated by standing in the cold room. This would suggest that inactivation on standing in the cold room and inactivation by prolonged freezing are the same phenomenon in principle, especially since after thawing and heating for 5 minutes at 60°C., the activity was found to be the same as for the unfrozen but heated solution (triangles and circles on curve *A* of Fig. 3).

In accord with this it was found that the solution actually must remain in the frozen state for a certain length of time before a noticeable inactivation occurs. The manipulation of freezing and immediate thawing in itself did not change the activity. Fig. 4 shows that this inactivation after 48 hours in the frozen state is considerably greater than after 24 hours. The "potential" activity in both cases is the same however, since upon thawing and subsequent heating for 5 minutes at 60°C. approximately the same activity was found. Inactivation by freezing therefore is completely reversible. Fig. 5 illustrates the influence of

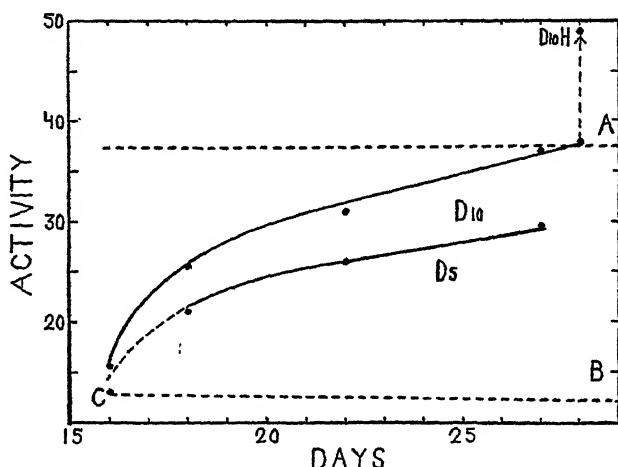


FIG. 5. Relative urease activity of a crude water extract of jack bean meal 1:15, expressed in micromols ammonia formed per milliliter reaction mixture at 20°C. Influence of dilution. Curves A and B, same as in Fig. 3. C, activity of the extract after 16 days' standing at 2-5 °C. Curve D5, diluted solution 1:5 standing at 2°C. Curve D10, diluted solution 1:10 standing at 2°C. D10H, activity of the 10 times diluted solution after 12 days' standing and subsequent heating for 5 minutes at 60°C.

dilution on the activity. After 16 days in the cold room the crude aqueous extract was diluted 5 times and 10 times with distilled water and these dilutions were then kept in the cold room at the same temperature as the undiluted solution. For activity determinations 0.5 and 1.0 ml. respectively were taken. The activity of the undiluted extract was approximately constant during the period of the experiment (curve B). The diluted solutions, however, showed a marked increase in activity. This increase was greater the higher the dilution. The tenfold diluted solution (curve D10), after 12 days' standing and without heating had an activity equal to the heated control and gave after 5 minutes' heating an activity even considerably higher than this value (point D10H). This shows once more that the highest activity is obtained when a dilute solu-

tion is heated moderately. In this way and under the conditions of the experiment, an activity of 3 to 4 times that of the undiluted unheated solution could be obtained. Finally, Fig. 6 shows the effect of several hours' standing at 20°C. of the undiluted extract, subsequent to 24 days' storage in the cold room. A marked increase in activity occurred, although after 4 hours this increase was only about one-fourth of the increase obtained by 5 minutes' heating at 60°C.

In a previous experiment it was found that the activity after 48 hours' standing at room temperature, was approximately the same as after 24 hours and did not differ appreciably from the activity after 4 hours' standing at room

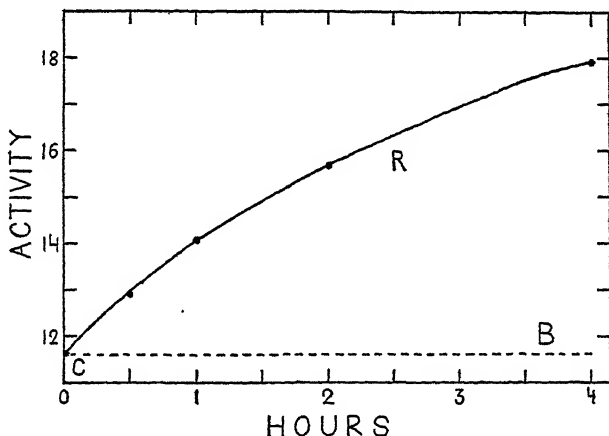


FIG. 6. Relative urease activity of a crude water extract of jack bean meal 1:15, expressed in micromols ammonia formed per milliliter reaction mixture at 20°C. C, activity after 24 days' standing at 2°C. Curve B, same as in Fig. 3. Curve R, activity during standing at 20°C.

temperature. This would suggest that the lower the temperature of heating the less is the increase in activity that can be obtained, in spite of continued heating. It would mean that any given dilution has a maximum of activity which is determined by the temperature; the higher the temperature the higher this maximum and the shorter the time for this maximum to be reached.

In summarizing the results of these experiments with a crude water extract it can be said that essentially the same conclusions may be drawn as from the experiments with crystalline urease. Furthermore, the evidence indicates that the same forces are responsible for the reversible inactivation brought about by standing at temperatures above or below the freezing point.

DISCUSSION

In view of the findings in the previous sections the possibility arises that urease molecules in solution tend to associate with each other or with other

protein molecules and in this process active groups are involved. These association forces oppose the random distribution of single molecules and not until these two forces come to equilibrium is a constant activity obtained. By assuming that the two opposing forces are of the same order of magnitude it would follow that the attainment of this equilibrium would be relatively slow and would be easily influenced by heat and by dilution.

Since reversible inactivation occurs by prolonged freezing, it must be assumed also that this association proceeds even in a solidified solution. In view of the fact that enzyme reactions do proceed in the frozen state, the above assumption does not seem unlikely, since according to present concepts the mechanism of enzyme reactions involves an association of enzyme and substrate.

Increase in activity by moderate heating and a partly reversible inactivation by freezing were observed in solutions of crystalline urease in distilled water as well as in those to which a protecting agent was added. The unprotected solutions do not give a constant "potential" activity over periods of several weeks, but the addition of another protein as a protector does not change the phenomenon in principle, although a foreign protein may possibly influence the process of activation and inactivation. It was found for instance, that the stock solution of urease in 2 per cent serum albumin (Fig. 2) after having reached a constant activity at 2°C., did not give a further decrease in activity by freezing for 24 hours. Also an enhancement of the inactivation in the crude water extract (Fig. 3) by precipitating out of proteins may not be excluded, even though urease itself is not precipitated as shown by a constant "potential" activity of the supernatant after centrifugation. In terms of the proposed association theory this would mean that association of urease molecules could more or less be prevented by other proteins.

Sumner *et al.* (7) found that twice recrystallized urease is not homogeneous in the ultracentrifuge, which might also be explained by assuming an association of urease molecules. This would be similar to the association of l-amino acid oxidase molecules as pointed out by Blanchard *et al.* (8). These authors noted that their highly purified and electrophoretically homogeneous enzyme was not homogeneous in the ultracentrifuge and consisted of 2 molecular species one 4 times as heavy as the other. The two components did not show a difference in activity however and the association apparently did not involve active groups. If the non-homogeneity of urease in the ultracentrifuge were caused by this same phenomenon, then a moderately heated solution should show a smaller average particle weight. However, it is hard to imagine how further dilution of solutions which are already very dilute could cause a breaking up of aggregates. This could better be visualized by assuming that in a relatively dilute solution the molecules do not actually form complexes, but rather tend to orient towards each other or, at the most, form a kind of loose structure. Theoretically, this would imply that at any definite temperature at which heat denaturation does not yet occur, a maximum activity could only be reached at

infinite dilution. Such more or less speculative assumptions should be supported by experiments which actually show whether association or dissociation is taking place, such as molecular weight determinations or by electron micrographs.

Finally, it might be pointed out that previous measurements of urease activity involving changes in temperature, such as estimations of the activation energy, where the foregoing factors were not controlled, are subject to reexamination. It would be indeed interesting to investigate whether under appropriate conditions similar phenomena might not also affect the activity of other enzymes.

SUMMARY

1. It has been shown that the activity of solutions of twice recrystallized urease is reversibly increased by moderate heating and reversibly decreased by storage in the cold, even in the frozen state.

2. Crude extracts of jack bean meal containing potent urease undergo this same type of reversible activation by heating and inactivation by cooling. Dilution has the same potentiating effect on the activity as moderate heating.

As much as a fivefold increase in activity can be obtained when a sample previously inactivated by storage for 24 hours at -10°C . is heated for 5 minutes at 60°C .

3. Solutions of crystalline urease protected by serum albumin and preserved in the cold give a constant "potential" activity over a period of more than 30 days if heated 5 minutes at 60°C . before assay.

4. The data presented have been interpreted to mean that an association between urease molecules (or between urease and other proteins) might occur, resulting in inactivation of the enzyme which would be reversed on dissociation.

5. It has been postulated that the same forces are responsible for the reversible inactivation brought about by standing at temperatures above or below the freezing point.

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THE RELATIONS OF THE PLASMA MEMBRANE, VITELLINE MEMBRANE, AND JELLY IN THE EGG OF NEREIS LIMBATA

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INTRODUCTION

It is recognized that the surface of a cell may be covered with one or more extraneous coats in addition to the plasma membrane. Certain of these layers in marine egg cells are variously called vitelline membrane, chorion, jelly hull, etc. (Chambers, 1940). Since certain ova can develop after removal of these extraneous layers (including the vitelline membrane), it is clear that the control of cell permeability lies in the much thinner, and invisible, plasma membrane (or protoplasmic egg surface). So called naked cells are those covered only by the plasma membrane. It is obviously of importance to know the relation of the plasma membrane to the other cortical structures of the cell.

Lillie (1911) described a cortical layer of the unfertilized *Nereis* egg as consisting of alveoli with homogeneous contents, the alveolar walls being continuous internally with the egg protoplasm, and uniting externally to form a protoplasmic layer (plasma membrane) applied to the vitelline membrane (Fig. 1 A). This cortical layer is about 7 microns thick in an egg of 140 microns diameter. With the release, at fertilization, of the alveolar contents to form the external jelly layer, the alveolar walls remain as delicate strands of protoplasm uniting the vitelline membrane to the surface of the egg, and the contracted alveoli of the cortical layer constitute the perivitelline space (Fig. 1 A'). Lillie does not state whether these protoplasmic strands traversing the perivitelline space are eventually retracted into the egg substance across the perivitelline space. Presumably some permanent protoplasmic connections must persist between the plasma membrane and the body of the egg protoplasm. On the basis of his observations, Lillie regarded the perivitelline space as *intraovular* (i.e., intracytoplasmic), with a delicate external cytoplasmic wall (the plasma membrane) lining the vitelline membrane. Because of its position, he considered the plasma membrane comparable in some respects to the fertilization membrane of the echinoderms.

Chambers (1933) offered an alternative interpretation of the cortical structure of the unfertilized *Nereis* egg. He suggested that the jelly precursor, instead of being intraovular, is extraprotoplasmic and lies between numerous protoplasmic processes or strands which extend from the egg surface to the enveloping vitelline membrane (Fig. 1 B). The spermatozoon, according to Chambers, becomes attached at fertilization to one or several of these protoplasmic strands. As the jelly is extruded the strands persist, broaden, and coalesce to form the sperm entrance cone. A flow of cytoplasm enlarges this cone, but the sperm head does not enter through the mem-

brane until about 45 minutes after contact. When the sperm entrance cone begins to withdraw into the egg there is an accompanying withdrawal of all cytoplasmic extensions lying across the perivitelline space. The egg surface (plasma membrane) then becomes evenly contoured, leaving a perivitelline space between it and the vitelline membrane (Fig. 1 *B'*). Chambers considers the protoplasmic strands of the unfertilized egg to be more numerous than those of the fertilized egg before retraction of the strands has taken place.

Novikoff (1939) reinvestigated the surface changes at fertilization of the *Nereis* egg. He describes the layer of cortical jelly precursor granules as a homogeneous intraovular layer without alveolar walls, which he considers to be an optical illusion. He failed to find any definite protoplasmic filaments in the unfertilized *Nereis* egg but describes the behavior of the hyaline protoplasmic filaments which appear following

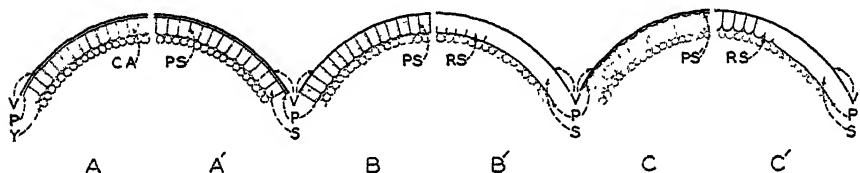


FIG. 1. Sectors of the cortex of the *Nereis* egg before (*A, B, C*) and after fertilization (*A', B', C'*) according to Lillie (*A-A'*), Chambers (*B-B'*), and Novikoff (*C-C'*).

Explanation of symbols: *CA*, cortical alveoli of jelly precursor granules; *P*, plasma membrane; *PS*, protoplasmic strands; *PS'*, points of origin of protoplasmic strands; *RS*, retracting protoplasmic strands; *S*, perivitelline space; *V*, vitelline membrane; *Y*, yolk spheres. In *A'* the walls of the empty alveoli represent the protoplasmic strands. In *C* the plasma membrane is represented as it is beginning to separate from the vitelline membrane immediately following activation of the egg. The points of attachment of the plasma membrane to the vitelline membrane which (according to Novikoff) account for the origin of the protoplasmic strands, are apparent at this time.

insemination. These filaments, according to Novikoff, are hyaline strands formed at points where the cortical protoplasm adheres to the vitelline membrane as the latter is separated from the egg surface (Fig. 1 *C*). These filaments eventually are withdrawn as the egg assumes an even contour during the period of egg movements described by Lillie (1912) and by Hoadley (1934). Novikoff conceives of the protoplasmic strands of the fertilized egg, not as the walls of emptied alveoli, as Lillie suggested, but as the deeply indented surface of the protoplasm (Fig. 1 *C'*). To ascertain whether the jelly precursor of the unfertilized *Nereis* egg is intraovular (as Lillie believed) or extraprotoplasmic (as Chambers suggested), Novikoff performed the following experiments. When unfertilized eggs are placed in isotonic NaCl at pH 9.6,¹ the vitelline membrane swells from the egg surface and, with gentle agitation of the container, breaks at one point and the "naked" egg rolls out. Novikoff observed no jelly on the surface of such denuded eggs, when tested by examination in a Chinese ink suspension

¹ It has been pointed out elsewhere (Costello, 1945) that this solution probably had a pH of 10.5.

and by rolling several eggs together into intimate contact. He observed no change in the structure of the cortical layer of the eggs that survive the alkaline NaCl treatment. From these observations he concluded that the jelly must be intraovular since an exudation of jelly is to be expected upon removal of the vitelline membrane if the jelly precursor granules are lying packed between the plasma membrane and the vitelline membrane. When sperm were added to the denuded *Nereis* eggs, Novikoff observed

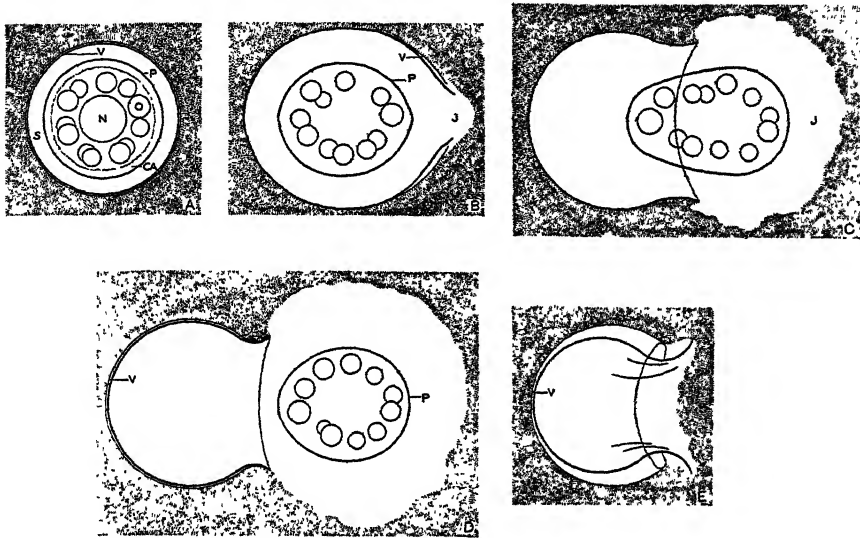


Fig. 2. Unfertilized egg of *Nereis* placed in alkaline sodium chloride to which Higgins India ink has been added to indicate presence or absence of jelly. A, 2 minutes after treatment; B, about 20 minutes after treatment; C, 24 minutes after treatment; D, 25 minutes after treatment; E, empty double membrane after escape of ovum. In A, germinal vesicle as well as oil droplets is visible. In B, C, and D, the germinal vesicle is no longer visible. This indicates egg activation.

Explanation of symbols: CA, cortical "alveolar" layer; J, jelly; N, germinal vesicle; P, plasma membrane; S, jelly-filled perivitelline space; V, vitelline membrane (double, consisting of thin outer and thick inner components).

jelly extrusion by these eggs, without sign of visible filaments, and concluded that contact between egg and vitelline membrane is essential for filament production.

However, Novikoff's observations are incomplete, and the evidence is erroneous on which he bases his conclusion (which appears to be correct) that the jelly precursor granules are intraovular.

The present reinvestigation of the effects of alkaline NaCl (pH 10.3-10.5) indicates, in brief summary, the following: (1) The lifting of the vitelline membrane from the surface of the unfertilized egg of *Nereis* by alkaline sodium chloride is due to the swelling of jelly under this membrane (Fig. 2 A-D), as

has been briefly described by Costello and Young (1939 *a, b*) and by Costello (1945). (2) After the denuded egg has rolled free of the membrane, jelly can be demonstrated in the empty membrane by means of India ink (Fig. 2 *E*) or Chinese ink, or by hand-controlled micro needles. (3) Jelly can be demonstrated also around the denuded egg as it escapes from the membrane (Fig. 2 *D*), but this jelly gradually dissolves as the egg remains in the alkaline sodium chloride. (4) The cortex of an unfertilized egg from which the vitelline membrane has been removed by alkaline sodium chloride treatment is, in some cases, thinner than that of a normal, untreated, non-fertilized egg. In the majority of these eggs the cortex has disappeared entirely and the germinal vesicle has ruptured by the time the egg is free of its membrane (Fig. 2 *B-D*). (5) Some eggs do not survive the treatment with alkaline sodium chloride. These are eggs in which the cortex violently releases all the jelly stored in the jelly precursor granules, and in which the plasma membrane is apparently destroyed. (6) A large proportion of the more intact, denuded, unfertilized eggs, when returned to sea water, slowly release their remaining jelly, and proceed to partial development at the slow tempo characteristic of parthenogenesis. Adding sperm to intact, denuded, unfertilized eggs does not increase the percentage showing jelly extrusion, nor change the tempo of development. If a sperm suspension is added to denuded eggs from which the clear, transparent jelly is being extruded, the sperm will clearly outline the practically invisible jelly by accumulating at its periphery. (7) Alkaline NaCl produces no increase in width of the perivitelline space of *Arbacia*, *Asterias*, or *Chaetopterus* eggs, which normally do not extrude jelly at the time of insemination.

If we accept Novikoff's observations, we are unable to account for the mechanism of membrane removal by alkaline sodium chloride. A reconsideration of the question of the location of the plasma membrane in relation to the jelly precursor granules is therefore essential.

Evidence from Centrifuging

An experimental test of the relation of the jelly precursor granules to the plasma membrane is provided by centrifuging the unfertilized *Nereis* egg with sufficient force to displace these granules. If the granules form an integral stratum of the centrifuged egg, they are intraovular. If they occupy a region separated from the main body of the protoplasm by a membrane boundary (as do the test cells of the centrifuged *Styela* egg), they are extraovular. Since the jelly precursor granules disappear from the egg cortex following fertilization, to give rise to the jelly lying external to the vitelline membrane, a comparison of eggs centrifuged before and after jelly extrusion will identify with certainty those granules in the centrifuged unfertilized egg. As described by Costello (1940), if unfertilized eggs are centrifuged for 60 minutes at 66,000 times gravity, most of the cortical material occupies the heaviest stratum of

the egg, no trace of an alveolar arrangement remaining (Fig. 3). This is the stratum of small granules which contains the vortex of neutral red-staining granules (Fig. 3 C and Fig. 4 A). In eggs centrifuged 25 minutes after insemination, this stratum is lacking, and the erythrophilous granules no longer form a vortex, but accumulate in a narrow layer at the extreme centrifugal pole (Fig. 4 B, C, D). The second hyaline zone, which lies between the yolk stratum and the neutral red-staining granules, is considerably wider in the

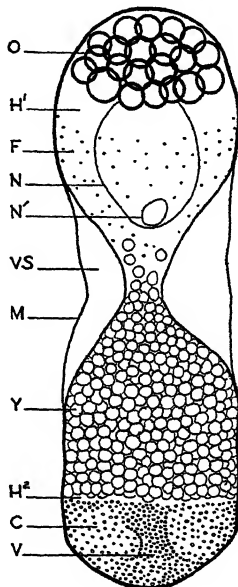


FIG. 3. Unfertilized egg of *Nereis limbata* centrifuged for 60 minutes at 66,000 times gravity. Sketched about 5 minutes after centrifuging. *O*, centripetal zone of oil droplets; *H*¹, upper hyaline zone; *F*, zone of fine granules which stain with neutral red; *N*, germinal vesicle (oocyte nucleus); *N*¹, nucleolus; *V**S*, vacuolar space (cytolysis vacuole) where vitelline membrane has pulled away from protoplasmic surface; *M*, vitelline membrane; *Y*, zone of yolk spheres; *H*², second hyaline zone; *C*, zone of cortical granules; *V*, vortex of erythrophilous granules.

fertilized egg (Fig. 4 B, C, D). The jelly precursor granules of the centrifuged unfertilized egg (Fig. 3, stratum C, or Fig. 4 A) are entirely lacking in the centrifuged fertilized egg (Fig. 4 B, C, D). The dark-field photomicrographs of the centrifuged fertilized eggs (Fig. 4 C, D) show clearly the finely granular material of the lower half of the hyaline zone which is practically invisible by bright-field illumination. The refractile, lipid-like appearance of the centrifugal zone of erythrophilous granules is also indicated. Unfortunately, we have no adequate criterion for identifying the mitochondrial layer of these centrifuged eggs.

An examination of the centrifuged unfertilized egg (Fig. 3 and Fig. 4 A) makes it clear that the stratum of the cortical granules with the contained vortex of erythrophilous granules is actually an integral part of the protoplasm.

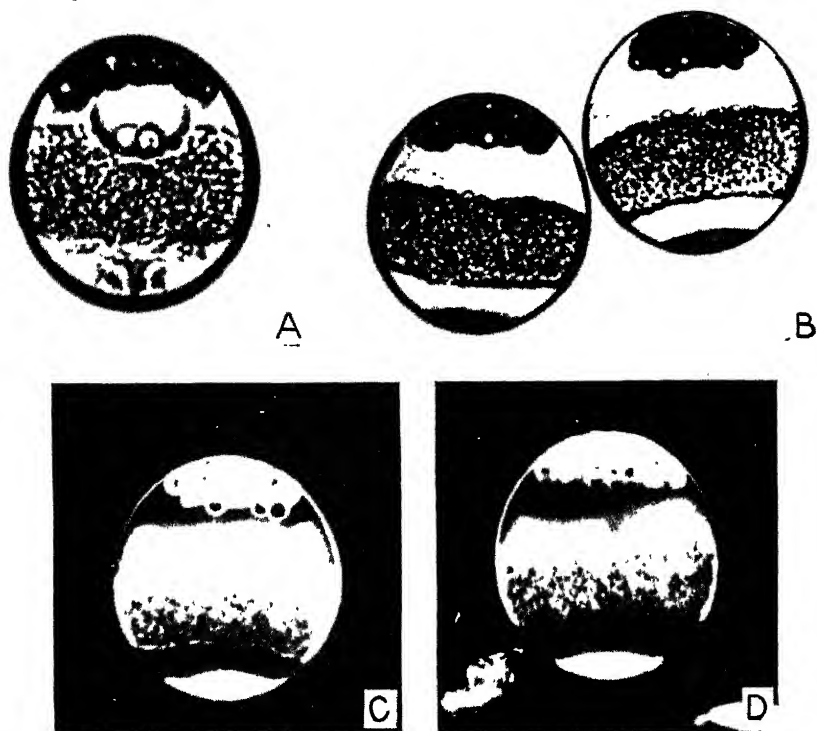


FIG. 4. Photomicrographs of: A, unfertilized egg centrifuged 7 minutes at 140,000 times gravity, photographed 4 minutes after centrifuging. The unfertilized egg has two centrifugally driven nucleoli in its nucleus, a very unusual condition. B, Eggs centrifuged 5 minutes at 140,000 times gravity, 64 minutes after insemination, photographed 5 minutes after centrifuging. Note that there is a wide second hyaline zone just above the centrifugal zone of erythrophilous granules in the fertilized egg. The jelly precursor granules occupy this stratum of the centrifuged unfertilized egg, with the included erythrophilous granules in a vortex. C and D, same as B, but by dark-field illumination. $\times 250$.

Therefore, these granules must have been intraovular before, as well as after, centrifuging. Raven (1938), using the eggs of *Nereis dumerilii*, describes a centrifugal zone of cortical material which stains brick red with brilliant kresylviolett in less completely stratified unfertilized eggs. He states that this zone is missing in centrifuged fertilized eggs, in which he identifies only

three zones. Presumably the neutral red-staining granules are sedimented only at higher forces than those used by Raven.

The fact that the vitelline membrane may separate from the protoplasmic surface to produce a vacuolar space (Fig. 3) at the neck of the "dumb-bell" under the influence of prolonged but relatively low centrifugal forces is further evidence that the two membranes (vitelline and plasma) are normally in intimate contact. Such vacuolar spaces are large and asymmetrical in more strongly centrifuged eggs. The unfertilized eggs do not pinch in two across the neck of this dumb-bell, despite the fact that the length of the egg cylinder is more than three times its diameter. This must indicate that the laws of surface tension for fluid cylinders as used by Harvey (1931) are inapplicable here. Centrifuged eggs showing such vacuolar spaces are not activated, and will show a cortical response to spermatozoa when inseminated (Costello, 1940). The asymmetry of this cortical response (jelly extrusion) in the centrifuged egg will be described in a later paper.

It is probable that the vacuolar space in the centrifuged eggs is not filled with jelly or perivitelline colloid, since upon standing after centrifuging, the "dumb-bells" tend to return to a more spherical form, with marked decrease in the volume of the vacuolar space. When tested in a Chinese ink suspension, there is no jelly extruded through the vitelline membrane until the egg is inseminated. Thus the decrease in volume of the space must be due merely to loss of water and salts to which the vitelline membrane is permeable. Eggs deformed to the dumb-bell shape by centrifuging never fully regain their original shape, but remain as oblate spheroids.

These vacuolar spaces of centrifuged eggs bear a superficial resemblance to the clear "cytolysis vacuoles" which are often seen on the surface of non-centrifuged eggs and result from local injury. These cytolysis vacuoles represent areas of the cortex where the jelly precursor granules have broken down to secrete their colloidal materials between the plasma membrane and the vitelline membrane. If the cytolysis due to the local injury has extended beyond the cortical layer of jelly precursor granules, the "cytolysis vacuole" may contain the granular debris of disintegrated yolk spheres. Cytolysis with yolk granule breakdown involves a considerable intake of water, and the vacuole may form as a large blister at the egg surface. Cytolysis vacuoles are the result of localized pressure injury, and the released jelly usually fails to pass through the vitelline membrane, as shown by testing with Chinese ink.

Evidence from Experimental Membrane Elevation

A second experimental test of the relation of the perivitelline space to the plasma membrane is provided by applying the alkaline sodium chloride membrane removal technique of Hatt (1931, 1932) and Novikoff (1938, 1939) to

unfertilized and fertilized *Nereis* eggs. The details of the technique are given by Costello (1945).

The vitelline membrane of the *Nereis* egg, which appears as a single membrane, can be shown experimentally to consist of two parts—a thin outer layer and a thick inner layer. Upon treatment with alkaline sodium chloride (pH 10.3–10.5), these layers, under some circumstances, may be separately elevated from the protoplasmic surface of unfertilized eggs (Fig. 2 *A–E*). The ease with which the separation of the two components of the double membrane is accomplished differs for different lots of eggs. Frequently the two layers are simultaneously elevated as one membrane and may or may not later split apart. The fact that the fertilized egg may develop normally (Costello, 1945) after both portions of this double membrane are lifted (Fig. 7 *E*) or removed is evidence that there is a plasma membrane remaining.

The first stage of membrane elevation of unfertilized eggs in alkaline sodium chloride is characterized by a crenation of the protoplasmic surface under the vitelline membrane. This is undoubtedly due to many points of attachment between the protoplasmic surface and vitelline membrane. Later the protoplasmic surface smooths off and becomes evenly contoured (Fig. 2 *A*, 7 *A*, *B*, *D*). As these coarse elevations retract to the protoplasmic surface, they may leave behind fine, practically invisible filaments traversing the perivitelline space. These fine filaments disappear also as the perivitelline space widens. Fig. 2 illustrates the sequence of events following treatment of the egg with alkaline sodium chloride after the initial crenation has disappeared. In an abundant quantity of alkaline sodium chloride, the entire process of denuding the egg occupies only about 15 to 20 minutes. In the egg sketched, the germinal vesicle disappeared during the course of the treatment (Fig. 2 *B*). This is a sign of parthenogenetic activation. The cause of membrane elevation is as follows: (*a*) The membrane is either rendered impermeable to the jelly by the alkaline sodium chloride, or the jelly molecules are changed in size or other properties so that they can no longer pass through the membrane. (*b*) A jelly precursor beneath the vitelline membrane is caused to swell (*i.e.*, is activated) by the alkaline sodium chloride, thus elevating the membrane by the accumulation of the swelling jelly in the perivitelline space (Fig. 2 *A*, *B*). When double membranes are visible (Fig. 2 *A*, *B*, *D*, *E*), this may be due either to a material which swells between the two layers of the vitelline membrane, or to the fact that the innermost layer of the membrane is permeable to the jelly. The latter is more probable.

The proof that membrane elevation is caused by swelling of the jelly is: (*a*) Pressure with micro needles on the partially elevated membrane produces distortion of the egg protoplasm. (*b*) Upon rupture of the membrane by continued swelling, the presence of jelly around the emerging egg and within the membrane may be demonstrated by Chinese or India ink (Fig. 2 *B*, *C*, *D*) and

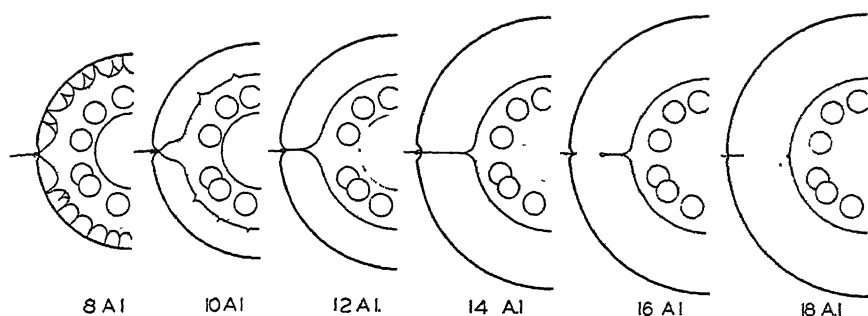


FIG. 5. *Nereis* egg placed in alkaline NaCl (pH 10.5) 5 minutes after insemination, and sketched (with camera lucida) at 2 minute intervals beginning 8 minutes after insemination (A. I.). Note exaggerated sperm entrance cone and points of attachment of plasma membrane to vitelline membrane (8 A. I.); smoothing off of egg surface (10-12 A. I.); indentation of elevating vitelline membrane by head of sperm (10-14 A. I.); increasing width of perivitelline space due to accumulation of jelly therein; penetration of sperm head through membrane (16 A. I.); fusion of sperm head with egg surface (18 A. I.).

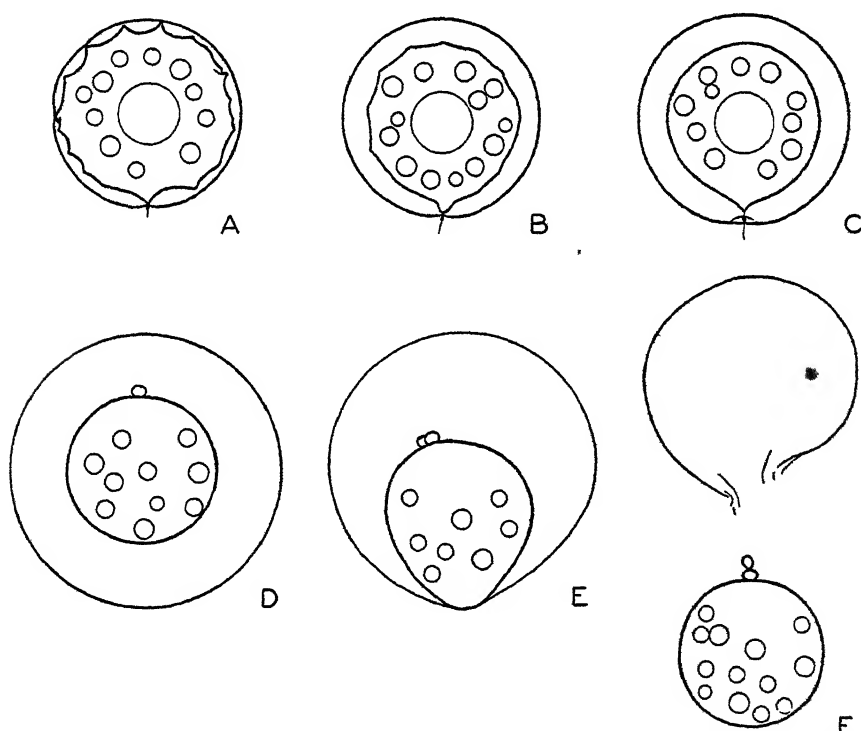


FIG. 6. *Nereis* egg placed in alkaline NaCl 5 minutes after insemination, and egg outlines redrawn from photomicrographs taken at intervals. A, 2 minutes after treatment with alkaline NaCl; B, 6 minutes later; C, 2 minutes later; D, 18 minutes later; E, 27 minutes later; F, 2 minutes later.

by manipulation with micro needles. Chinese ink is stable in sea water as well as in the alkaline solution, whereas India ink maintains its finely suspended state only in alkaline NaCl. (c) The jelly is absent from the outside of the membrane as long as the membrane is intact (Fig. 2 A). (d) Elevated membranes may be collapsed back against the egg surface by concentrated gum arabic solutions in sea water (Fig. 7 C, F), or by 2 per cent egg albumen in sea water, but not readily by hypertonic salt solutions. This indicates that the vitelline membrane after treatment with alkaline NaCl is permeable to water and relatively permeable to salts, but not to the perivitelline colloid or to the gum arabic or albumen. To produce this membrane collapse, the concentration of the external colloid must be sufficiently great to provide an osmotic pressure exceeding that of the perivitelline colloid. However, this experiment demonstrates only that the perivitelline material is a colloid, not that it is a gel. When the egg is placed in these solutions which exert colloidal osmotic pressure, the membrane does not contract smoothly against the egg surface, but wrinkles and forms folds, without any particular pattern. In this respect it resembles a cellophane bag from which the contents have been removed, rather than a rubber balloon from which the air has been released. This indicates that the membrane had been stretched beyond its limits of elasticity, and that there has been a molecular reorientation during the distention. The partial closure of the empty membrane after release of the ovum (Fig. 2 E, Fig. 6 F) indicates, however, that a small amount of residual elasticity remains after the membrane has been distended to the breaking point. Chambers (1942) describes the fertilization membrane of the *Arbacia* egg as being non-elastic below the dimensions it possesses at any period during its normal expansive growth. The vitelline membrane of the egg of *Nereis* may be described as being relatively inelastic below the dimensions it assumes during its distention in alkaline sodium chloride. Under normal conditions, in the absence of treatment with alkaline NaCl, the vitelline membrane is permeable to the jelly which is extruded through it following insemination. (e) If eggs are placed in alkaline sodium chloride at varying times after insemination, the width of the perivitelline space produced is inversely proportional to the amount of jelly already extruded. That is, if the eggs are treated 5 or 10 minutes after insemination (Figs. 5 and 6), the membranes elevate and rupture, liberating the eggs; whereas at 30, 40, or 60 minutes after insemination, the membranes elevate only a little, and do not rupture to free the eggs. When the fertilized eggs are introduced into alkaline sodium chloride shortly after insemination, the initial visible effect is a marked crenation just under the vitelline membrane (Figs. 5, 6 A). This crenation is somewhat more marked than in the case of the unfertilized egg, and persists longer. Points of attachment between the protoplasmic egg surface and the vitelline membrane must therefore still exist. The perivitelline space at this time (5 to 10 minutes after insemination),

when the egg is introduced into alkaline NaCl, therefore must be incomplete, and traversed by the connecting filaments. When the jelly is prevented from leaving the perivitelline space by the alkaline NaCl treatment, it swells there, and the connecting filaments which produce the points of the crenations are gradually broken (Fig. 5, 8 A.I.-10 A.I., Fig. 6 A), to give a smooth surface to the protoplasm below the perivitelline space, just as in the unfertilized egg. The only filament remaining is the sperm entrance cone filament, which becomes greatly stretched (Fig. 5, 10 A.I.-14 A.I.) as the perivitelline space widens.

Novikoff postulates an enormous stretching of the plasma membrane to form the protoplasmic strands. There is an alternative view that the protoplasmic strands are composed of some "cement substance" which attaches the plasma membrane to the vitelline membrane. This substance might also form the material of the sperm entrance cone. There appears to be, at present, no adequate basis for deciding between these two alternatives. If the plasma membrane itself is stretched to more than three times its normal surface area, one might expect a drastic alteration in permeability during the initial stages of egg activation. Just (1928) records that during the first 25 minutes after insemination, the *Nereis* egg is most susceptible to extreme hypotony.

In the light of the evidence presented, Lillie is undoubtedly correct in stating that the jelly precursor granules of the unfertilized egg are intraovular, but incorrect in stating that the perivitelline space of the fertilized egg is also intraovular. Chambers' suggestion that the jelly precursor granules of the unfertilized egg are extraovular is not tenable. Novikoff's view that the jelly precursor granules are intraovular and that the material of these granules must pass through the plasma membrane which then retracts from the vitelline membrane, leaving the perivitelline space between the two, is in agreement with my interpretation.

It is important to emphasize the fact that there are no visible pores in the vitelline membrane through which the jelly is normally extruded on activation of the *Nereis* egg (Just, 1928). If the jelly precursor granules are intraovular, activation of the *Nereis* egg must result in the release from the jelly precursor granules of micellae which can pass through both the plasma membrane and the vitelline membrane. Swelling (or polymerization) of these molecules may begin between the plasma membrane and the vitelline membrane, with concomitant intake of water, to elevate the vitelline membrane sufficiently to produce the perivitelline space, but the great majority of these molecules or micellae, in normal fertilization, apparently are able to pass out through the vitelline membrane before they swell to produce the thick external jelly layer. It seems reasonable to assume that swelling of the released jelly precursor molecules is conditioned by the presence of calcium and hydroxyl ions in the sea water. Costello (1945) has pointed out that neutral isosmotic calcium

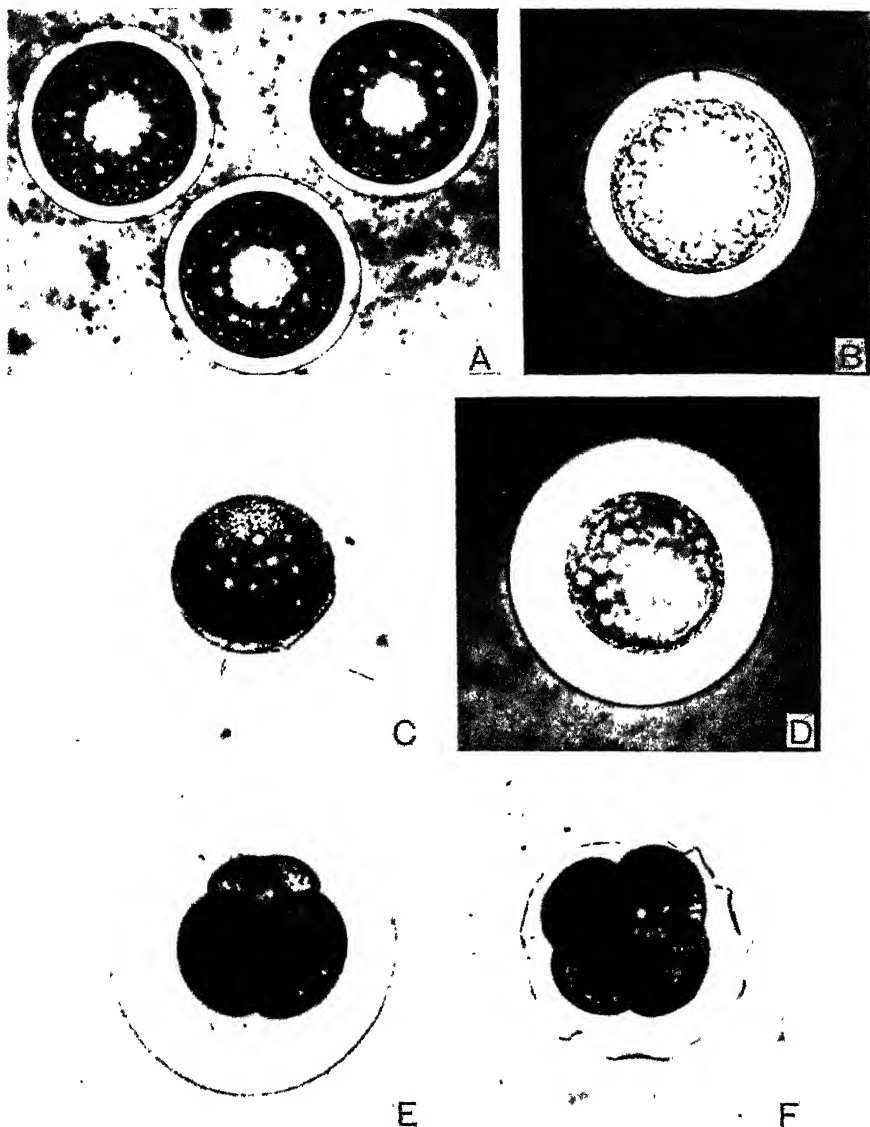


FIG. 7 *A*. Photomicrograph of uninseminated *Nereis* eggs placed in alkaline sodium chloride with Chinese ink and photographed 5 minutes later. Note that no jelly has exuded through the vitelline membrane, which is now separated from the egg surface by a perivitelline space. The central clearer region of the egg marks the germinal vesicle.

FIG. 7 *B*. Photomicrograph of uninseminated egg treated in same manner as in Fig. 7 *A*, photographed after 13 minutes in alkaline sodium chloride.

(Legend continued on following page)

chloride is as effective an agent for producing membrane elevation as is alkaline sodium chloride. In terms of the experimental membrane elevation this may mean that the jelly precursor molecules must pass through the membrane before swelling or they cannot pass through at all. This, then, is in harmony with the simpler of the two above suggested explanations of the effect of alkaline NaCl; *i.e.*, an effect of the hydroxyl ions on the jelly rather than on the membrane. This effect on the jelly is the explanation preferred by Redfield and Bright (1921) for the exaggerated membrane elevation produced in *Nereis* eggs by radiations. This explanation replaced their earlier view (1918) that radiations produce an abnormally thick fertilization membrane (swelling of the membrane due to intake of water) on the *Nereis* egg.

Since the evidence upon which the above conclusions are based depends upon the behavior of the jelly, a section is appended on the nature of this interesting material.

The Nature of the Jelly

Lillie (1911, 1912) stated that the vitelline membrane of the egg of *Nereis* is permeable to both colloids and crystalloids. Just (1928) states that no satisfactory hypothesis has ever been presented to explain how the jelly is able to pass through the vitelline membrane. It has been pointed out above that if the jelly precursor micellae are intraovular they must pass through not only the vitelline membrane but also the plasma membrane. The assumption that the plasma membrane is permeable to the jelly precursor molecules and impermeable to ordinary salts is difficult to accept on the basis of a pore theory, since non-hydrated jelly molecules (whether of the mucoprotein or carbohydrate type of jelly) would presumably be of greater molecular volume than

FIG. 7 C. Photomicrograph of uninseminated *Nereis* egg left in alkaline sodium chloride for 26 minutes (until membrane was elevated to a distance intermediate between that of Figs. 7 B and D), then transferred to a concentrated solution of gum arabic, and photographed 5 minutes after transfer. Note that the perivitelline space has disappeared and that the vitelline membrane has collapsed in wrinkles against the egg surface.

FIG. 7 D. Photomicrograph of uninseminated *Nereis* egg treated with alkaline sodium chloride plus Chinese ink for 47 minutes.

FIG. 7 E. Photomicrograph of *Nereis* egg treated 4 minutes after insemination with alkaline sodium chloride which was neutralized in acid sodium chloride 46 minutes later. The egg was then permitted to develop in sea water, and photographed 108 minutes after insemination, at the 4-cell stage. Note polar bodies on micromere and normal arrangement of blastomeres.

FIG. 7 F. Photomicrograph. History same as that of Fig. 7 E except placed in gum arabic 143 minutes after insemination, and photographed (at 8-cell stage) 14 minutes later. The membrane is partially collapsed and wrinkled. $\times 150$.

salt molecules. This difficulty might not exist if it were a question of partition coefficients rather than pores. The properties of the jelly substance are therefore of paramount importance.

To ascertain the nature of the *Nereis* egg jelly Dr. John Ferry (personal communication, 1939) performed a series of preliminary analyses on the external jelly collected from the fertilized eggs of five *Nereis* females. He found that it contains less than 1 per cent nitrogen, and suggests that a more accurate analysis of a carefully purified sample might show it to be nitrogen-free. It is at least 75 per cent carbohydrate (estimated by the Tillmans-Philippi method, and corrected for ash), and contains uronic acid. The evidence suggests that it is a uronic acid polymer, occurring as the calcium (and perhaps magnesium) salt. Prolonged dialysis at neutral reactions failed to remove the inorganic material, giving a product with an ash content of 57 per cent. Subsequent electrodialysis reduced the ash content to 13 per cent, leaving the pH about 5.0. If the latter ash represented calcium oxide which was stoichiometrically combined, this would lead to an equivalent weight of about 190, which is about that of uronic acid. There was insufficient material to confirm the hypothesis that there is one calcium ion combined with each pair of uronic acid residues. The jelly is precipitated in fibrous form by barium ions at alkaline reactions, but not at neutrality. The jelly from the eggs of the five females contained only about 17 mg. of ash-free substance.

Costello and Lavin (1943) pointed out that the jelly layer of the fertilized *Nereis* egg absorbs a negligible amount of ultraviolet light of wave length 2537 Å as compared with the absorption by the yolk spheres. In the centrifuged unfertilized eggs the zone of cortical granules absorbs very much less ultraviolet light of this wave length than does the zone of yolk spheres. The wave length 2537 Å is the region of maximum absorption for typical proteins and nucleic acids. According to Spek (1934) the yolk spheres are albuminoid in composition.

According to Tyler (1942) the nitrogen content of *Arbacia* egg jelly is about 5 per cent. Tyler states that this is rather low for a simple protein and since there is evidence for the presence of polysaccharide, it might be a glycoprotein. Ferry (1939) studied the properties of two other animal jellies—one surrounding the eggs of *Arenicola cristata*, the other from fresh adults of *Mnemiopsis leidyi*. The *Arenicola* egg jelly was shown to be a polysaccharide containing uronic acid, while the *Mnemiopsis* jelly was a mucoprotein containing 12 per cent nitrogen. *Nereis* egg jelly is apparently similar to the *Arenicola* egg jelly.

SUMMARY

1. The problem of the relation of the plasma membrane to the extraneous coats and cortex of the *Nereis* egg is discussed in the light of the observations of Lillie, Chambers, and Novikoff.

2. Evidence obtained from experiments with the centrifuge, and by treating eggs with alkaline sodium chloride, indicates that the plasma membrane of the unfertilized egg is external to the jelly precursor granules of the cortex.

3. Experiments with alkaline sodium chloride indicate that the perivitelline space of the fertilized egg is extraovular after jelly extrusion is complete.

4. The cortical behavior (membrane elevation) of the *Nereis* egg in alkaline sodium chloride and the cortical response (jelly extrusion) following activation of the egg in normal fertilization or parthenogenesis are attributed largely to the properties of the jelly, and presumably, to its reactions with calcium and hydroxyl ions.

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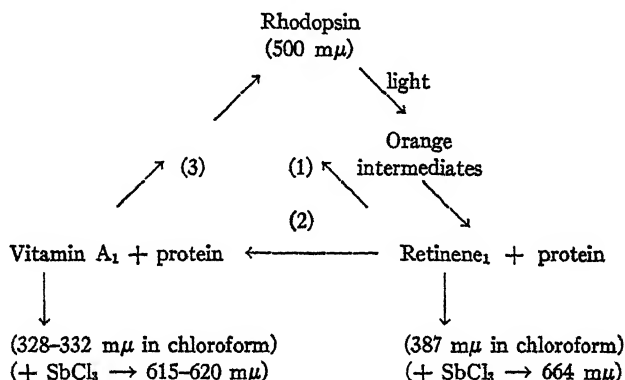
THE REDUCTION OF RETINENE₁ TO VITAMIN A₁ IN VITRO*

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The rhodopsin system in the rods of the vertebrate retina operates as a complete cycle only in the intact eye. Here the light-sensitive rhodopsin participates with the carotenoids retinene₁ and vitamin A₁ in a closed chain of reactions of the following skeletal form:



One has only to remove the retina proper from contact with the underlying tissues which line the optic cup to abolish the synthesis of rhodopsin from vitamin A₁ (reaction (3) above). According to Kühne this process requires the cooperation of a living pigment epithelium (Ewald and Kühne, 1878, page 255; Kühne, 1879).

If the system is further disintegrated by bringing rhodopsin into solution in aqueous digitonin, ordinarily processes (1) and (2) are also virtually eliminated. Nothing remains but the complex succession of reactions initiated by light which transform rhodopsin into retinene₁ and protein.

The present investigation is concerned with reaction (2), the conversion of retinene₁ to vitamin A₁. An indication of its nature is provided in the development initiated by Morton of Liverpool. Morton and his coworkers found that on mild oxidation vitamin A₁ is transformed into a product which resembles retinene₁ in spectrum and antimony chloride reaction (Ball, Goodwin, and Morton, 1946). They have presented evidence that this product is an aldehyde;

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they believe it to be vitamin A₁ aldehyde. We have confirmed and extended these observations (Wald, 1947-48). Retinene₁, whether synthetic or from the retina, possesses a conjugated carbonyl group; and partition experiments, still unpublished, show that it has lost the primary hydroxyl group present in vitamin A₁. There is therefore good reason to suppose that one change in going from vitamin A₁ to retinene₁ is the oxidation of the alcohol radical to aldehyde.

The further correspondence between the synthetic substance and natural preparations of retinene₁ is not wholly complete; specifically, crude natural retinene₁ is a pH indicator while the synthetic substance is not. There can be no doubt however that both substances stand in the most intimate chemical relationship. It seems clear therefore that the conversion of retinene₁ to vitamin A₁ is or includes the reduction of the carbonyl group of retinene₁ to hydroxyl.

I

Cattle Retina Powder

In 1942-43 one of us succeeded in bringing the system which converts retinene₁ to vitamin A₁ into a cell-free preparation (Wald, 1947, 1948). Cattle retinas were frozen, desiccated in high vacuum, ground to a fine powder, and exhaustively extracted with petroleum ether, all in darkness. The residue, a dry powder which contained unaltered rhodopsin, was stirred into a brei with neutral phosphate buffer. On exposing this to light, the rhodopsin was bleached, and the retinene₁ formed from it was converted almost quantitatively to vitamin A₁. These experiments were interrupted by the war, and have only recently been resumed.

Such an experiment is described below; the results are shown in Figs. 1 and 2.¹

Experiment.—Twenty cattle retinas were removed from the eyes in dim red light and were frozen at once in solid CO₂. They were desiccated at this low temperature

¹ The spectra shown in Figs. 2, 4, and 7 to 12 were drawn by the recording photoelectric spectrophotometer of Hardy, and have simply been mounted for publication. This instrument has special advantages for measuring the antimony chloride reaction with retinene and vitamin A. It is very sparing of light, an important consideration since it is now known that the blue products formed by both carotenoids with antimony chloride are highly photosensitive (*cf.* Wald, 1947-48). These products also fade even in darkness, and the speed of the Hardy instrument is therefore also advantageous. In our tests, 2.3 ml. of a saturated solution of antimony chloride in chloroform is added to 1 ml. of a chloroform solution of the test sample, with the absorption cell already in position in the spectrophotometer, and the recording is completed within about 1 minute.

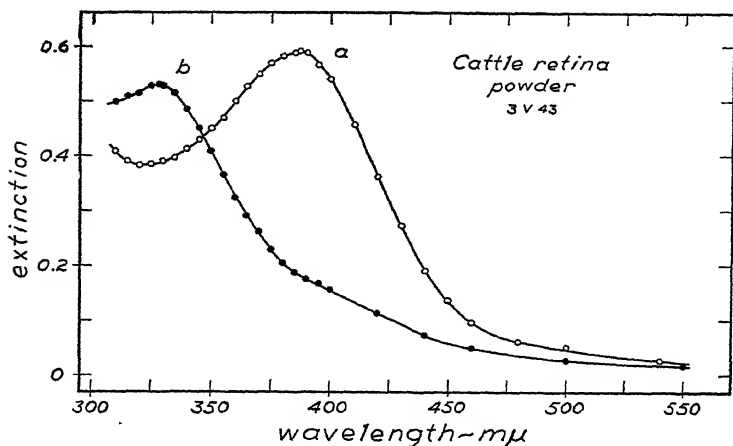


FIG. 1. The conversion of retinene₁ to vitamin A₁ in a cell-free brei from cattle retinas. In half this preparation the rhodopsin was destroyed in the dark with methanol at the beginning of the procedure; its extract displays the retinene₁ band in chloroform at 387 mμ (curve *a*). The other half was bleached with light and let stand 2 hours before being treated similarly; here the retinene₁ band is almost wholly replaced by that of vitamin A₁ at about 330 mμ (curve *b*). Ordinates are plotted as extinction or optical density, $\log I_0/I$, in which I_0 is the incident and I the transmitted intensity.

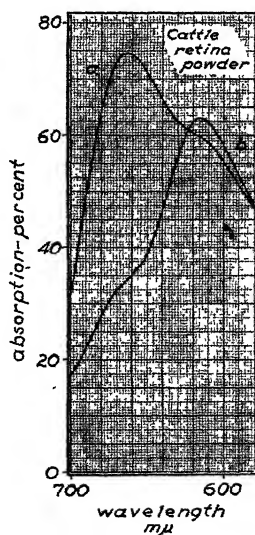


FIG. 2. The conversion of retinene₁ to vitamin A₁ in a cell-free brei from cattle retinas. Spectra of antimony chloride tests with the extracts of Fig. 1. The control preparation yields the absorption band at 664 mμ characteristic of retinene₁; the hump in the region of 610 mμ always accompanies this and does not signify vitamin A₁ (curve *a*). In the preparation bleached by light and incubated before extraction, the retinene₁ band is almost completely replaced by the vitamin A₁ band at 615 mμ; a trace of residual retinene₁ is also apparent (curve *b*).

overnight under high vacuum. The dry tissue was ground in a mortar, and divided into two equal portions. Each portion was extracted in a Soxhlet apparatus with low boiling petroleum ether (b.p. 20–40°) for 1½ hours in darkness. The solid residues were stirred in 5 ml. M/15 phosphate buffer, pH 6.95.

To one such brei 10 ml. of methanol was added in the dark. This was the control. The added methanol destroys the rhodopsin, liberating retinene₁, and blocks all further transformations.

Both portions were exposed to bright light, and were left in moderate light for 2 hours at room temperature. Then 10 ml. of methanol was added also to the second, experimental brei. Both were centrifuged, the solid material dehydrated by grinding with anhydrous sodium sulfate, and extracted with low boiling petroleum ether in the Soxhlet apparatus for 2 hours.

Both extracts were transferred to chloroform. Their spectra, measured with the Beckman spectrophotometer, are shown in Fig. 1. The control displays the retinene₁ maximum at 387 mμ; in the brei which had been bleached and incubated before methanol was added this is replaced by the vitamin A₁ band at about 330 mμ.

Both extracts were concentrated in chloroform and samples mixed with antimony chloride (a saturated solution in chloroform). The spectra of the resulting blue products are shown in Fig. 2. The control preparation displays the retinene₁-antimony chloride band at 664 mμ, the experimental the vitamin A₁-antimony chloride band at about 615 mμ.

The conversion of retinene₁ to vitamin A₁ in this brei was all but complete; only a trace of retinene₁ is evident in curves *b* of both figures.

II

Fresh Rhodopsin Solutions

Some time ago it was shown that fresh aqueous solutions of frog rhodopsin exhibit a special type of bleaching which goes further than is observed in the same solutions after a period of aging (Wald, 1937–38). This is one of the thermal or “dark” reactions which follow the exposure of rhodopsin to light. It was called dark process III in the analysis of the bleaching of rhodopsin in solution.

Bliss (1948) has now reported that the basis of this change is the formation of vitamin A₁. There is much else in this paper with which we do not agree, particularly the rôle assigned to what Bliss calls “acid indicator yellow” and its supposed relations with retinene₁. We have however confirmed the formation of vitamin A₁ in fresh solutions of frog rhodopsin.

An example of this reaction is described below. It can be traced directly in the original solution through the spectral changes which follow exposure to light (Fig. 3); and is further established through the antimony chloride tests with extracts of such solutions (Fig. 4).

There has been some confusion in the literature regarding the ordinary course of bleaching of rhodopsin in solution, the nature of the products formed, and

the relation of these events to what happens in the intact retina. It will aid in understanding what follows to review this situation briefly.

On irradiation of rhodopsin in neutral solution at room temperature, the first product sufficiently stable to be measured spectrophotometrically is orange

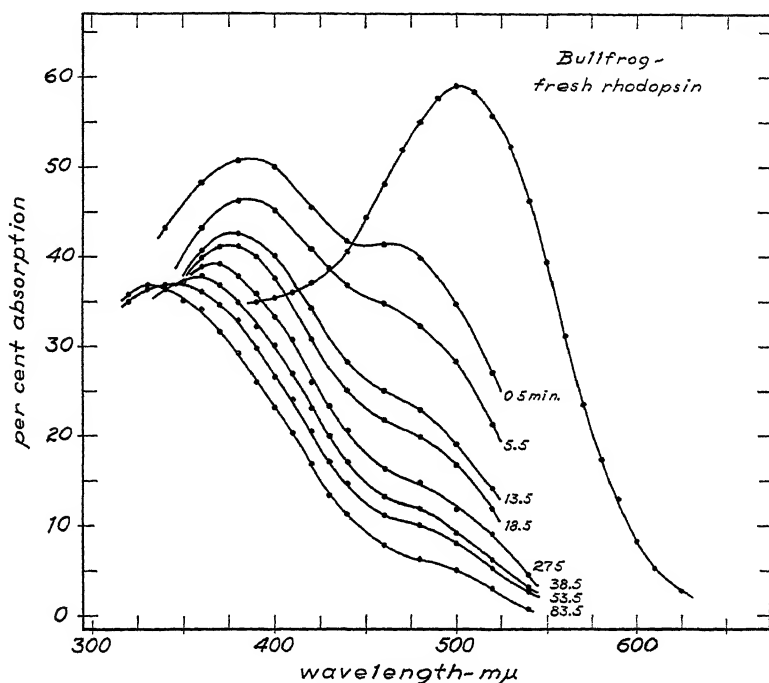


FIG. 3. Conversion of retinene₁ to vitamin A₁ in a fresh aqueous extract of bullfrog retinas. The original solution, protected from light during preparation and measurement, displays the rhodopsin maximum at 502 mμ. It was exposed to light for 25 seconds, and spectra thereafter measured in darkness at intervals (minutes) indicated at the right of the curves. The initial bleached spectrum shows the band of the orange intermediate at 480 mμ, superimposed on the retinene₁ band at 385 mμ. The 480 mμ absorption fades leaving finally a low absorption at about 500 mμ due to a trace of regenerated rhodopsin. The retinene₁ band gives way to a final maximum at 330 mμ due to vitamin A₁. Extract from bullfrog retinas in 1 per cent digitonin; pH 6.8; 23°C.

in color. It possesses a high absorption band in the retinene₁ position at about 385 mμ, and a broad hump in the visible spectrum at about 480 mμ. In darkness following the exposure to light the 480 mμ absorption falls, while the 385 mμ absorption simultaneously rises (Wald, 1937-38; dark process II).

The highly unstable material responsible for the absorption at 480 mμ is in-

cluded in what Lythgoe called "transient orange" (Lythgoe and Quilliam, 1938). We have not adopted this term because we believe it to designate not a single molecular species but a complex of intermediates between rhodopsin and retinene₁ + protein. Some and perhaps all of these substances change in spectrum with pH. Nor have we inserted this term heretofore in the equations of the rhodopsin cycle, because of its equivocal character, and because the status of orange intermediates in the retina as contrasted with solutions is still obscure (*cf.* Wald, 1937-38, page 828).

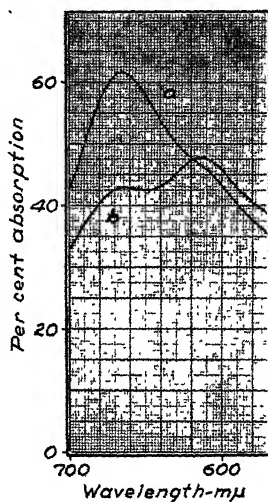


FIG. 4. Conversion of retinene₁ to vitamin A₁ in a fresh aqueous extract of bullfrog retinas. Spectra of the antimony chloride reaction with extracts of the final bleached product shown in Fig. 3 and of a control portion of the same solution destroyed in the dark with methanol. The control solution yields the retinene₁ band at 664 mμ (curve *a*); in the bleached and incubated solution this has largely been replaced by the vitamin A₁ band at about 615 mμ (curve *b*).

The final product of bleaching rhodopsin in aged solutions is a mixture of retinene₁ and protein, for the most part still loosely coupled together in a way that scarcely alters the retinene₁ spectrum. The experiments which led to this conclusion were presented some time ago (Wald, 1937-38, pages 812-813). In the equations of the rhodopsin cycle, however, we have not written retinene₁ as coupled to protein because in the retina, unlike aqueous solutions, retinene₁ is entirely liberated from protein by bleaching (Wald, 1935-36).

The retinene₁-protein which results from bleaching rhodopsin in solution is almost colorless when alkaline and bright yellow when acid (Chase, 1935-36).

For this reason Lythgoe called it "indicator yellow." We find its absorption maxima to lie at about $366\text{ m}\mu$ at pH 9–9.5, $387\text{ m}\mu$ at pH 6.7–7, and $393\text{ m}\mu$ at pH 4–4.5. The pH lability of this complex does not depend ultimately on the coupling of retinene₁ to protein; for protein-free retinene₁, extracted with fat solvents from bleached retinas or rhodopsin solutions and brought back into aqueous solution with such a detergent as digitonin, still is a pH indicator. On the other hand the synthetic product manufactured from vitamin A₁ has lost this property; as has also natural retinene₁ partly purified by adsorption and elution (Wald, 1947–48).

Lythgoe described as the acid form of "indicator yellow" a material possessing a broad absorption band maximal at about $440\text{ m}\mu$. When rhodopsin is bleached at pH about 4 this material appears as an initial product. In light or darkness the $440\text{ m}\mu$ maximum slowly moves toward shorter wavelengths, finally coming to rest at about $390\text{ m}\mu$, the maximum of acidic retinene₁ (Wald, 1937–38). The $440\text{ m}\mu$ material therefore is not acidic retinene₁-protein but its precursor; and so is homologous with the $480\text{ m}\mu$ precursor of retinene₁ in neutral solution. In Lythgoe's terminology it should be regarded as part of the "transient orange" complex, not as the acidic form of "indicator yellow."

Having once obtained retinene₁-protein by bleaching rhodopsin in solution, one can by treatment with strong acids convert it to highly colored products with absorption maxima at $440\text{ m}\mu$ or longer wavelengths. The same can be done with retinene₁ extracted from such solutions or from retinas with fat solvents and therefore protein-free; even here retinene₁ may still be coupled with other molecules. Ball *et al.* (1948) have reported obtaining such products from synthetic retinene₁ on treatment with acids in the presence of certain proteins, amino acids, and aromatic amines. These are the artefacts which Bliss (1948) calls "acid indicator yellow." They have an interest of their own, but are neither precursors of vitamin A₁ nor do they play any other direct rôle in the visual processes.

The bleaching of a fresh solution of neutral rhodopsin is shown in Fig. 3. The original spectrum, measured before exposure to light, possesses the rhodopsin maximum at $500\text{ m}\mu$. Immediately following irradiation the spectrum displays the broad maximum at about $480\text{ m}\mu$ of the orange intermediate, superimposed on a high retinene₁ band at about $385\text{ m}\mu$. In darkness, the $480\text{ m}\mu$ maximum slowly declines, leaving finally a very low absorption at about $500\text{ m}\mu$ due to a little regenerated rhodopsin. Instead of the $385\text{ m}\mu$ maximum simultaneously growing, as it would have done in aged solutions, it moves toward shorter and shorter wavelengths, reaching a final position at about $330\text{ m}\mu$. This is the band of vitamin A₁. In the final spectrum a raised absorption in the region of $380\text{ m}\mu$ marks a residue of unchanged retinene₁.

Antimony chloride tests with extracts of such solutions confirm the trans-

formation of retinene₁ to vitamin A₁. Half of the original preparation which yielded Fig. 3 had been kept dark as a control, and was destroyed in the dark with methanol. Its extract, mixed with antimony chloride, yielded curve *a* of Fig. 4, displaying the 664 mμ maximum of retinene₁ alone. The final product of the experiment shown in Fig. 3 was treated similarly. It yielded curve *b* of Fig. 4, showing beside a residue of retinene₁ the dominant absorption at 615 mμ due to newly formed vitamin A₁.

Experiment².—Retinas of two bullfrogs were extracted by stirring in 2 per cent aqueous digitonin solution for 30 minutes. The mixture was centrifuged 15 minutes at 11,500 R.P.M. and the clear extract poured off. This was diluted with an equal volume of M/15 phosphate buffer, pH 6.84, and divided into two equal portions. All these operations were carried out in dim red light.

Half the solution was set aside in the dark as a control; the remainder was used in the experiment shown in Fig. 3. The latter was irradiated 3 hours after the beginning of dissection and 1½ hours after the beginning of extraction. Spectra were measured with the Beckman spectrophotometer, a slower instrument than is desirable for work of this type. Nevertheless the spectra show clearly the principal events which follow exposure of the rhodopsin to light.

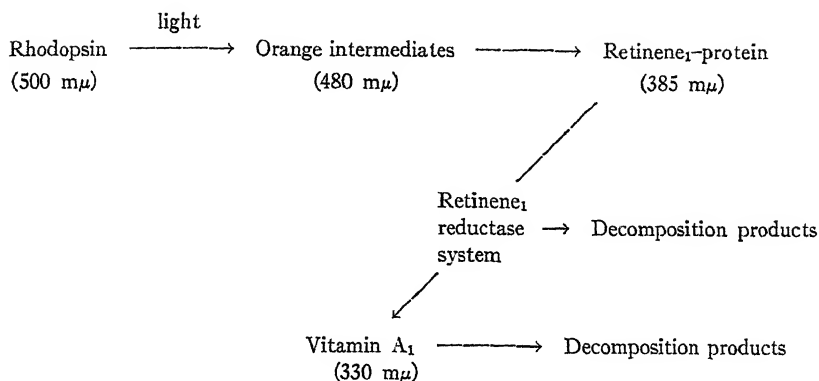
For the antimony chloride tests shown in Fig. 4, methanol was added to a concentration of 60 per cent to both the bleached solution and the dark control. Both solutions were extracted by shaking with petroleum ether. The extracts were transferred to chloroform and mixed with antimony chloride solution in the spectrophotometer. The spectra of the blue products were recorded within the first minute after mixing the reagents.

Fresh solutions of rhodopsin exhibit a further change, not shown in Figs. 3 and 4. This is the slow destruction of vitamin A₁. Within 10 hours after the exposure to light, all carotenoids have usually disappeared from the solution. The direct spectrum shows no evidence of either retinene₁ or vitamin A₁, and the antimony chloride test is negative.

Finally, in such fresh solutions the enzyme system itself disintegrates. Within a period of several hours at room temperature the preparation loses most of its capacity to convert retinene₁ to vitamin A₁.

The changes known to occur in fresh rhodopsin solutions can therefore be formulated as follows:—

² In this and all subsequent experiments the frogs were dark-adapted overnight before removal of the retinas. The dissection and all other preparatory operations were performed either in dim red light, to which rhodopsin is insensitive, or in darkness. In each experiment these conditions were maintained to the point at which it is explicitly stated that solutions or tissues were exposed to light. In all cases this was the white light from a tungsten filament lamp.



The significance of the term "retinene₁ reductase system" will become evident below. This system alone presents a number of opportunities for side reactions.

In so complicated a mixture of interdependent processes, it is almost impossible to interpret reliably the effects of change of temperature, pH, or other conditions. Before one can examine with confidence the properties of the transformation of retinene₁ to vitamin A₁, it will be necessary to isolate this reaction to a reasonable degree and to stabilize its enzyme system.

III

Rod Outer Limbs

We have described the conversion of retinene₁ to vitamin A₁ in a cell-free brei and in aqueous detergent solutions. In order to analyze such systems further one would ordinarily fractionate them in the attempt to isolate their essential components. We had already begun such experiments, when the investigation took a new turn with the discovery that the enzyme system is already fractionated anatomically in the structure of the retinal rods.

The vertebrate rod is composed of two sections, the so called inner and outer limbs or segments. The inner limb is not very different from a nerve cell, though it makes synaptic connection only at one end since it is the first member in an excitation chain. This portion of the rod contains the nucleus and is presumably the seat of the main vegetative functions.

The outer limb is a complex cellular outgrowth, which contains all the rhodopsin to be found in the retina. Within a sheath of neurokeratin, it appears to be composed of alternate layers of protein and lipid, both highly oriented in what is essentially a crystalloidal structure (Schmidt, 1938). In the frog, in which the rods are unusually large, the outer segment is a cylinder about 6 to 9 micra wide and about 50 micra long. Within this small compass is enclosed the whole of the photoreceptor process. This must represent about as high a degree of isolation of a physiological function as the organism offers.



FIG. 5. Microphotograph of rod outer segments suspended in Ringer solution. Magnification about 500. The longitudinal striations which can be seen in most of the outer limbs are characteristic of fresh preparations, and probably are evidence of a fibrillar structure in the outer limb. Later, cross-striations appear and eventually dominate the structure; the first of these also are visible in the photograph.

If one removes the retina from a frog eye into Ringer solution with all possible care, the solution examined under a microscope is found to contain large numbers of isolated rod outer limbs. These have been broken off in the course of dissection, just at the juncture with the inner limbs. Thereafter they maintain their integrity for considerable periods (Fig. 5).

By special methods one can remove larger numbers of outer segments from the retina. Dense suspensions of them have been used by Lythgoe (1937) and by Saito (1938) to prepare rhodopsin solutions.

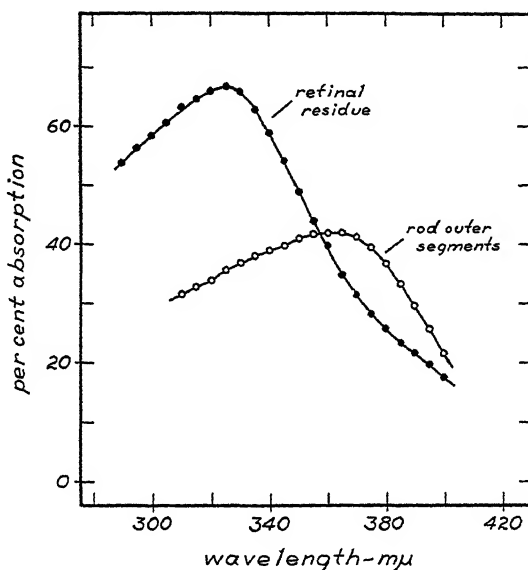


FIG. 6. Rod outer segments detached from the retina do not convert their retinene₁ to vitamin A₁. Numbers of rod outer segments had been scraped from frog retinas and left suspended with the retinal remainders during a period of bleaching and incubation. They were then isolated, and they and the retinal residues extracted separately with hexane. The spectra of these extracts are shown. The detached rods yield the band at about 365 μ m of retinene₁; the retinal residues to which outer limbs had remained attached yield the band at 325 μ m of vitamin A₁.

Such a suspension of rod outer limbs can be separated almost completely from other retinal tissues. When prepared under deep red light it contains a large quantity of rhodopsin. On exposure to white light this bleaches to a yellow-orange color. Unlike whole retinas, however, such isolated outer segments do not go on fading to colorlessness, the change associated with the conversion of retinene₁ to vitamin A₁. On extracting the bleached outer limbs even after hours of incubation one finds only retinene₁. The transformation of retinene₁ to vitamin A₁ does not occur in the isolated outer limb.

This situation is not changed if the outer limb is left suspended in the same medium with the remainder of the retina. It is enough to break the outer segment away from the underlying tissue to abolish its capacity to form vitamin A₁. On the other hand those outer limbs which remain attached to the retina continue to convert their retinene₁ to vitamin A₁ as before.

These relations are demonstrated in the following experiment, the results of which are shown in Fig. 6.

Rod Suspension.—The retinas of dark-adapted frogs (*Rana pipiens*) are prepared in Ringer solution in dim red light. They are scraped by gently stroking the posterior surface which bears the receptor cells with a spatula or fine forceps. The retinal remainders are lifted into a separate test tube after scraping; usually they still retain about half their original content of outer limbs. The suspension of isolated outer segments is filtered through three layers of cheesecloth to remove shreds of other tissues. The suspension is centrifuged, and the rods used as desired.

Experiment.—Outer limbs were scraped from the retinas of nine dark-adapted frogs. In this instance they were left suspended with the other retinal tissues in Ringer solution. The whole suspension was exposed to white light, then allowed to stand for 1 hour at room temperature. The detached outer limbs were now filtered from the retinal remainders. Both tissues were dehydrated by grinding with anhydrous sodium sulfate, and were extracted with hexane. The spectra of these extracts are shown in Fig. 6.

The detached outer limbs yielded the retinene₁ band at about 365 m μ in hexane, with just a suggestion of the vitamin A₁ maximum at 325 m μ . The retinal remainders yielded the vitamin A₁ band alone. That is, though both tissues were incubated in the same medium, only the rods which had remained attached to the underlying retinal tissues had formed vitamin A₁. Judging by the ratios of the absorptions of retinene₁ and vitamin A₁ in this experiment, about twice as many rods had remained attached to the retina as had been broken away.

IV

The Coenzyme of Retinene₁ Reduction

If whole retinas are thoroughly mashed with a glass rod in Ringer solution or phosphate buffer, almost all the outer limbs are detached from other tissues in the process. Yet the suspension which results efficiently converts retinene₁ to vitamin A₁. The breaking up of the retinal tissue by grinding releases substances which promote this process in the outer limbs.

Presumably these factors are carried to the outer limbs in solution in the suspension fluid. If such a suspension is centrifuged and the supernatant liquid poured off, the solid residue which contains all the rhodopsin has lost almost completely the power to form vitamin A₁. On re-adding the supernatant it regains this capacity (Fig. 8).

A clear, colorless water extract of retinas added to isolated rod outer limbs constitutes a complete system for converting retinene₁ to vitamin A₁. Indeed

it is not necessary for this that the rods maintain their normal structure. They may be frozen, desiccated, ground, and exhaustively extracted with petroleum ether; and the residue from this treatment, suspended in a water extract of

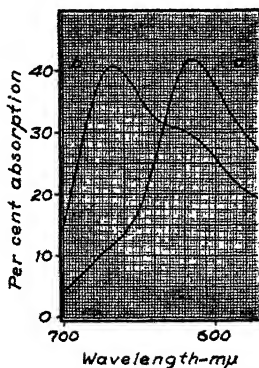


FIG. 7

FIG. 7. Rod outer segments suspended in a water extract of retina convert retinene₁ to vitamin A₁; the washed retinal residue does not. Outer limbs were isolated and were frozen, dried, and preextracted with petroleum ether. The portions of retina from which they were taken were extracted with neutral phosphate buffer, and the rod residues suspended in the extract. They and the washed retinal tissue were irradiated and incubated. The spectra of the antimony chloride reactions with their extracts are shown. That from the washed retinal tissue displays the band of retinene₁ (curve *b*); while the outer segment preparation suspended in retinal washings has converted its retinene₁ entirely to vitamin A₁ (curve *a*). This result is just the reverse of that shown in Fig. 6; the difference is that here a water extract has been transferred from the retinal residues to the detached outer limbs.

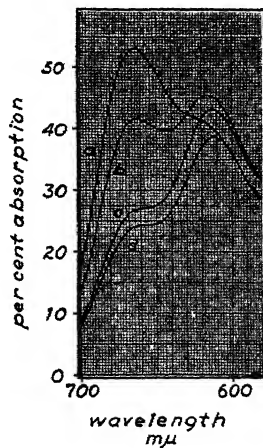


FIG. 8

FIG. 8. Washed retina is reactivated by returning the water extract; the water-soluble factor is relatively heat-stable. Washed retinal tissue had been suspended in a number of solutions, bleached, and incubated for 1 hour. The spectra of the antimony chloride tests with extracts of the final products are shown. The washed tissue suspended in phosphate buffer had not converted its retinene₁ to vitamin A₁ (*a*); suspended in a water extract of retina which had been held in boiling water for 7 minutes it performed about a half conversion (*b*); in retinal extract kept for 7 minutes on ice (*c*) or at room temperature (*d*) the conversion was nearly complete.

retina, still transforms its retinene₁ into vitamin A₁. On the other hand the retinal tissue from which the water extract was taken no longer can perform this conversion.

The following experiment, the results of which are shown in Fig. 7, demonstrates these relationships.

Experiment.—Retinas of ten dark-adapted frogs, dissected out under red light, were scraped as described above, and the rod outer limbs filtered from the retinal remainders. Both tissues were desiccated under vacuum at low temperature. The dried rods were extracted by shaking with three portions of petroleum ether, each time for 20 minutes. The dried retinal remainders were ground thoroughly with a glass rod in 1 ml. M/15 phosphate buffer, pH 6.8, for 15 minutes; then centrifuged for 15 minutes at 4000 R.P.M. The clear supernatant was poured off and used as a suspension medium for the outer limb residues. An equal volume of phosphate buffer was added to the washed retinal residues. Both mixtures were exposed to bright light for 1 minute, then were left for 1½ hours in moderate light at room temperature. Both suspensions were centrifuged and the solid material dehydrated by grinding with anhydrous sodium sulfate and extracted with 3.5 ml. chloroform. The extracts were concentrated and tested with antimony chloride. The spectra of the blue products are shown in Fig. 7. The rod powder suspended in a water extract of retina had converted its retinene₁ completely to vitamin A₁; while the washed retinal tissue had failed almost completely to perform this conversion.

The water extract of retina is highly unstable. It loses most of its activity within an hour at room temperature. On the other hand it does not lose all its activity on being brought to 100°C. for as long as 7 minutes, though this amount of heating should be enough to destroy most enzymes.

These relations are illustrated in the following experiment, the results of which are shown in Fig. 8.

Experiment.—Retinas of twelve frogs were dissected in red light and ground thoroughly for 15 minutes in 2 ml. of M/15 phosphate buffer, pH 6.84. The suspension was divided into four equal portions and all were centrifuged at 4000 R.P.M. The supernatants were poured off, mixed, and redivided into three equal portions. We had therefore prepared four portions of washed retina and three portions of retinal extract.

The latter were kept for 7 minutes, one on ice, one at 23°C., and the third in boiling water. All were brought to the same temperature. Then these extracts were added to three of the samples of washed retina, while to the fourth an equal volume of phosphate buffer was added. All were stirred and exposed together to white light, and were left at room temperature for 1 hour. All were centrifuged, the liquid poured off, and the solid residues dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride.

The spectra of the resulting blue products are shown in Fig. 8. The water extracts of retina which had been kept on ice (curve *c*) or at room temperature (curve *d*) displayed strong activity; in the washed retina to which they were added almost all the retinene₁ had been converted to vitamin A₁. The boiled extract also had been fairly active; here the retinene₁ was about half converted (curve *b*). The washed retinal tissue to which simple buffer had been added had failed to form vitamin A₁ (curve *a*).

The ease and completeness with which the water-soluble factors are washed from the retina and their relative stability toward heat argued against the

likelihood that they are enzymes. Our experiments suggested rather that we were dealing with relatively small and simple molecules, perhaps of the nature of substrates and coenzymes. In this case, however, there is no reason to expect these substances to be confined to the retina alone. One could look forward to finding them in other tissues.

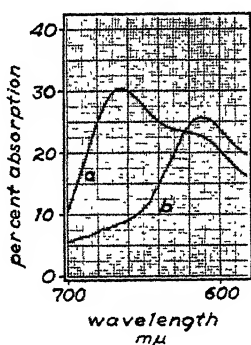


FIG. 9

FIG. 9. Boiled muscle juice reactivates washed retina. Equal portions of water-extracted retina were suspended in phosphate buffer and in a boiled juice of frog muscle. The suspensions were exposed to light, left at room temperature for 1 hour, and extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. The washed tissue in buffer solution did not convert its retinene₁ to vitamin A₁ (curve *a*); that suspended in boiled muscle juice did so completely (curve *b*).

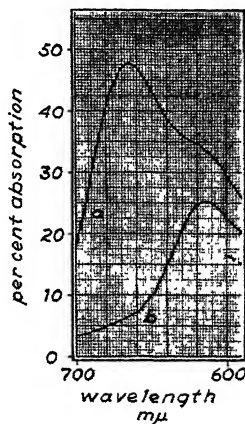


FIG. 10

FIG. 10. Boiled muscle juice activates isolated rod outer limbs. Equal portions of detached rod outer segments were suspended in phosphate buffer and in a boiled juice of frog muscle. The suspensions were exposed to light, left at room temperature for 1 hour, and the residues extracted with petroleum ether. The spectra of the antimony chloride tests with these extracts are shown. Outer limbs in buffer had failed to convert their retinene₁ to vitamin A₁ (curve *a*); those suspended in boiled muscle juice had done so completely (curve *b*). The relatively low content of vitamin A₁ shown in curve *b* is due to its destruction in preparations of this type.

To explore this possibility we prepared a boiled extract of frog muscle and added it to a preparation of washed retina. It promoted the conversion of retinene₁ to vitamin A₁ as efficiently as did the best of our retinal extracts. The boiled muscle juice was equally effective in promoting the formation of vitamin A₁ by isolated outer limbs. This left no doubt that the factors we sought are present in muscle as in retina, and are thermostable.

The action of boiled muscle juice on washed retina and on outer limbs is demonstrated in the following experiment and in Figs. 9 and 10.³

Experiment.—Retinas of twelve frogs prepared in red light were scraped thoroughly, the detached outer limbs filtered from the retinal remainders, and the latter ground in about 1.5 ml. of M/15 phosphate buffer, pH 6.84. The outer limb and retinal suspensions were each divided into two equal portions and centrifuged 15 minutes at 4000 R.P.M. The supernatants were discarded. This procedure netted us two portions of isolated outer limbs and two of washed retina.

A boiled muscle juice was prepared by mincing the gastrocnemius and sartorius muscles of one frog, and grinding them in a mortar with 10 ml. of a 1:1 mixture of Ringer solution and pH 6.84 phosphate buffer. This brei was filtered through cheesecloth, and the filtrate brought to a boil. The clear liquid was decanted and rapidly chilled to room temperature.

2 ml. of boiled muscle juice was added to one portion of outer limbs and to one of washed retina. To the remaining portions of both types of tissue was added 2 ml. of buffer-Ringer mixture. All four samples were stirred into suspension, exposed to bright white light, and left at room temperature for 1 hour. All were centrifuged, the liquid poured off, and the solid material dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride. The spectra of the blue products are shown in Figs. 9 and 10.

Washed retina suspended in boiled muscle juice converted its retinene₁ quantitatively to vitamin A₁ (Fig. 9, curve *b*); the same tissue in buffer solution failed to perform this conversion (curve *a*). Similarly, isolated outer limbs in boiled muscle juice transformed their retinene₁ completely to vitamin A₁ (Fig. 10, curve *b*), while they did not go through this reaction at all in buffer solution (curve *a*).

We have noted above that in the conversion of retinene₁ to vitamin A₁ an aldehyde group is reduced to hydroxyl. In this reaction therefore retinene₁ acts in the rôle of hydrogen acceptor. Boiled muscle juice contains a number of substances which might donate hydrogen for this process; it is famous also for its content of a major coenzyme of hydrogen transfer, cozymase, coenzyme I, or DPN.

A preparation of washed retina to which DPN was added still failed to convert retinene₁ to vitamin A₁. One would expect such a preparation to have retained at least a fraction of all its original enzymes; it may however have lost essential substrates.

³ Fig. 10 illustrates another relation of some interest. Retinene₁ is reasonably stable in suspensions of rod outer limbs; but when these are placed in circumstances in which they can form vitamin A₁, the vitamin is destroyed within several hours after its formation. This accounts for the low level of vitamin A₁ compared with retinene₁ shown in the figure. It will be recalled that a similar destruction of vitamin A₁ occurs in fresh rhodopsin solutions.

We therefore added DPN to washed retina combined with various concentrations of the potential hydrogen donors, lactic and succinic acids. In some instances small amounts of vitamin A₁ seem to have formed, but none of these preparations produced it efficiently.

Failing a suitable substrate temporarily, we tried the effect of already reduced cozymase (DPN-H₂). A sample of DPN was reduced with sodium hydrosulfite (Na₂S₂O₄), and the excess hydrosulfite oxidized away by blowing air through the solution (Green and Dewan, 1937). The reduced cozymase was added to a preparation of washed retina. It converted the retinene₁ formed on bleaching almost wholly to vitamin A₁. As might be expected of such a process, it was if anything aided by the total exclusion of oxygen.

The action of reduced DPN on washed retina is demonstrated in the following experiment and in Fig. 11.

Experiment.—Retinas of five frogs were prepared in red light and ground in M/15 phosphate buffer, pH 6.84. The suspension was divided into two equal parts and both were centrifuged 15 minutes at 4000 R.P.M. The fluid was discarded and the residues resuspended in 1 ml. of pH 6.84 buffer. These suspensions of washed retina were transferred to the bulbs of two Thunberg tubes.

Reduced DPN was prepared according to the method of Green and Dewan (1937). A commercial preparation of DPN (Schwarz, 60 per cent active) was made up in 5 ml. to a concentration of 0.15 per cent DPN. To this was added 2.5 ml. of a 1:1 mixture of 0.5 per cent NaHCO₃ and 0.2 per cent Na₂S₂O₄. The excess hydrosulfite was removed by bubbling air through the solution. A control solution was prepared by repeating the entire procedure with the same reagents but without DPN.

2.5 ml. of the reduced DPN and of the control mixture were placed in the bodies of the Thunberg tubes. The tubes were alternately evacuated and washed through with nitrogen three times and finally were closed under vacuum. The contents were then mixed and exposed to bright white light. They were left at room temperature in moderate light for 1¼ hours. The contents were then centrifuged, the residues dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride. The spectra of the blue products are shown in Fig. 11.

Washed retina suspended in the control salt mixture had failed to convert its retinene₁ to vitamin A₁ (curve *a*); but that to which reduced DPN was added had performed this conversion almost completely (curve *b*).

There remained the problem of finding a substrate which in the presence of washed retina or isolated rod outer limbs would reduce DPN. As noted above, neither succinic nor lactic acid fulfills this function. We have however found a first such substrate in fructosediphosphate.

A commercial preparation of hexosediphosphate promotes some conversion of retinene₁ to vitamin A₁ by washed retina or outer limbs even without the addition of DPN. Presumably this involves some residue of DPN retained by

the retinal tissue. But with added DPN the action goes much further, and yields a very efficient formation of the vitamin.

These relations are demonstrated in the following experiment, the results of which are shown in Fig. 12.

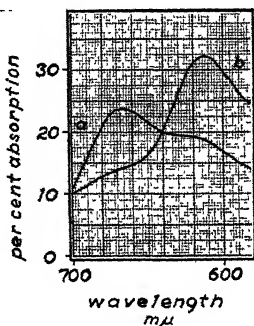


FIG. 11

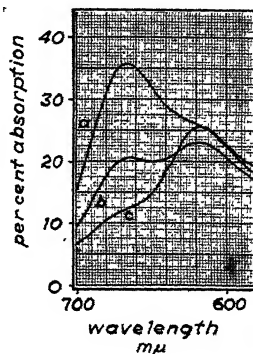


FIG. 12

FIG. 11. The action of reduced cozymase on washed retina. Equal portions of a preparation of water-extracted frog retina were suspended in a solution containing reduced DPN and in an otherwise identical solution lacking only the DPN. Both suspensions were bleached in the light, incubated, and the residues extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. The control preparation yielded retinene₁ alone (curve *a*); while in the washed retina to which reduced DPN had been added this had been converted almost completely to vitamin A₁ (curve *b*).

FIG. 12. The action of DPN and fructosediphosphate on isolated rod outer limbs. Equal portions of a preparation of rod outer segments were suspended in (*a*) phosphate buffer, pH 6.84; (*b*) a preparation containing fructosediphosphate in phosphate buffer; and (*c*) the latter mixture to which DPN was also added. The suspensions were made anaerobic, exposed to light, incubated for 2 hours, and the rod residues extracted with petroleum ether. Spectra of the antimony chloride tests with these extracts are shown. Rod outer limbs in buffer mixture yielded only retinene₁ (curve *a*); with hexosediphosphate added they had converted about half their retinene₁ to vitamin A₁ (curve *b*); with both hexosediphosphate and DPN added, the conversion to vitamin A₁ was nearly complete (curve *c*).

Experiment.—A suspension of rod outer limbs was isolated from the retinas of eleven frogs. It was divided into three equal portions. These were centrifuged, the liquid poured off, and the rods resuspended in 1 ml. portions of *m*/15 phosphate buffer, pH 6.84. The suspensions were transferred to the bulbs of three Thunberg tubes.

To the body of one Thunberg tube was added 2.25 ml. of the phosphate buffer as

control; to the second, 2 ml. of 0.017 molar fructosediphosphate:⁴ and 0.25 ml. of the phosphate buffer; and to the third tube this same mixture to which had been added 5 mg. of DPN powder (about 5×10^{-7} mols).

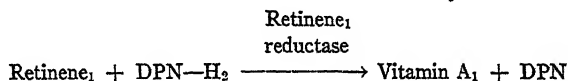
All the tubes were evacuated and flushed through with nitrogen several times, then shut off under vacuum. The contents were mixed, exposed to white light, and left in moderate light at room temperature for 2 hours. The contents were then centrifuged and the solid residues dehydrated by grinding with anhydrous sodium sulfate and extracted with petroleum ether. The extracts were transferred to chloroform and tested with antimony chloride. The spectra of the blue products are shown in Fig. 12.

Isolated rod outer limbs suspended in buffer mixture had as before failed to convert their retinene₁ to vitamin A₁ (curve *a*). In the presence of a crude preparation of fructosediphosphate they had performed a partial conversion (curve *b*). With fructosediphosphate and added DPN the conversion to vitamin A₁ was very nearly complete (curve *c*).

V

DISCUSSION AND CONCLUSIONS

We have shown that the conversion of retinene₁ to vitamin A₁ is a coupled reduction, for which cozymase (DPN) acts as coenzyme and fructosediphosphate can act as substrate. The essential process is the transfer of two atoms of hydrogen by DPN from a hydrogen donor to retinene₁, reducing its aldehyde group to the primary alcohol group of vitamin A₁:

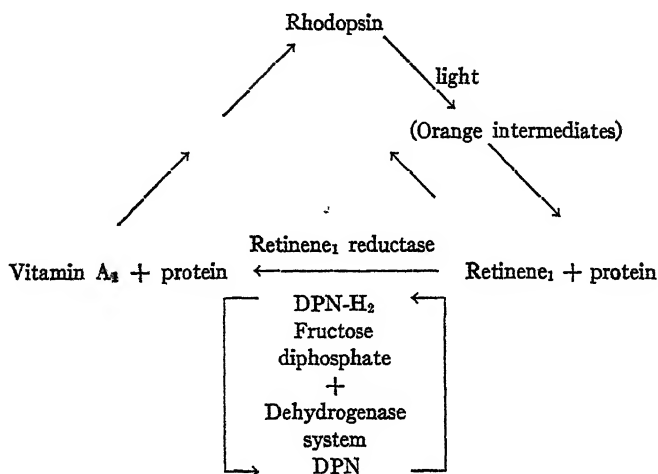


We have not yet demonstrated that the reduction of retinene₁ with DPN-H₂ requires a special enzyme, but this is made so probable by all that is known of such reactions that one is justified in assuming it to be the case. This apoenzyme, which we will call retinene₁ reductase, is present in the outer segment of the rod.

The system which reduces retinene₁ must work in conjunction with a second system which reduces DPN. This also requires a special apoenzyme which is

⁴ The fructosediphosphate used in these experiments was a preparation of the dibarium salt supplied by the Schwarz Laboratories of New York City. It contained 8.4 per cent P, and on this basis was about 83 per cent pure. To prepare it for use, it was taken up in HCl, the barium precipitated with sodium sulfate, and the solution neutralized with sodium hydroxide.

present in the rod outer limb. The organization of the total process can therefore be written:



This development lifts the rhodopsin cycle out of its former isolation, coupling it with one of the most general processes in cellular metabolism, the transfer of substrate hydrogen by cozymase. It introduces also a new vitamin relation. The rhodopsin system, long known to contain vitamin A₁ as a direct participant, is now seen to depend secondarily upon a member of the vitamin B complex, the antipellagra factor nicotinic acid amide, contained in DPN.

It is not necessary that fructosediphosphate, simply because it is effective, be regarded as the hydrogen donor in the reduction of retinene₁. The preparations of outer limbs or washed retina which catalyze this reaction probably are able also to degrade fructosediphosphate fermentatively, and one of its derivatives probably serves as the immediate source of hydrogen in our reaction. Among such possibilities one thinks first of 3-phosphoglyceraldehyde, the substance which reduces DPN in the alcohol and lactic acid fermentations, and the immediate product of the action of aldolase on fructosediphosphate.

Since the transformation of retinene₁ to vitamin A₁ is a reduction, place must be found elsewhere in the closed rhodopsin cycle for an oxidation. We have stressed heretofore the conjugation of carotenoid with protein in the synthesis of rhodopsin, and its cleavage from protein following the exposure of rhodopsin to light. It is now apparent that one or the other of these processes must include also an oxidative reaction.

This is not however the direct reoxidation of vitamin A₁ to retinene₁. A curious and significant property of the retinene₁ reductase system is that it operates irreversibly. In the intact isolated retina and in the *in vitro* systems we have described above, vitamin A₁ accumulates as the sole carotenoid end-prod-

uct. We have found no indication whatever of the oxidation of vitamin A_1 to retinene₁, except through the roundabout intermediation of rhodopsin in the complete retinal cycle.

It may be an advantage to the organism that the retinene₁ reductase system behaves in this fashion. Were vitamin A_1 oxidized directly to retinene₁, this would be a means of withdrawing the vitamin from circulation and accumulating large stores of retinene₁ for which there is probably no function outside the retina. It is an attractive hypothesis that in the retina the utilization of vitamin A_1 begins with its conjugation to the protein moiety of rhodopsin, and that it is oxidized only after this has occurred. This would then be a self-limiting process, restricted to the amount of rhodopsin protein present in the rod outer segment.

Since the rod outer segment contains retinene₁ and the apoenzymes for reducing both it and DPN, we may assume that in its normal position in the retina it contains also DPN and a suitable hydrogen donor. That is, it probably comes to lack DPN and substrates only after being detached from the rest of the retina. The isolated outer limb is no more than a fragment of a cell, broken off at one end. Such relatively small, water-soluble molecules as DPN and sugar derivatives probably leak from the detached outer segment into the suspension medium, until their concentrations have fallen too low to be effective. In this sense, the isolated outer limbs can themselves be regarded as a "washed" tissue. In the intact retina they probably contain all that is needed to reduce retinene₁. There is no present reason to believe that this process normally demands a migration of substances between the outer segments and the underlying retinal tissues.

It is now clear that the fresh rhodopsin solutions which are able without supplementation to convert their retinene₁ to vitamin A_1 must possess not only retinene₁ reductase and DPN, but the enzyme system and substrates for reducing DPN. The deterioration of such solutions could have multiple causes. Probably the primary cause of their decay, however, is the loss of DPN. It is now well known that this nucleotide is rapidly destroyed in autolysates from a wide variety of organs. Preparations of brain tissue, to which retina is closely related, are particularly active in this regard (Handler and Klein, 1942). We are now exploring the possibility that the reduction of retinene₁ can be stabilized in fresh solutions of rhodopsin and restored in aged solutions by the addition of DPN.

SUMMARY

In the surviving vertebrate retina the retinene₁ liberated by bleaching rhodopsin is converted quantitatively to vitamin A_1 . Recent chemical studies have indicated that in this process the aldehyde group of retinene₁ is reduced to the primary alcohol group of vitamin A_1 (Morton; Wald).

Some time ago we brought this reaction into a cell-free brei prepared from cattle retinas. The retinas were frozen, desiccated, ground, and exhaustively extracted with petroleum ether; the resulting powder, stirred in neutral buffer solution and exposed to light, converted its retinene₁ completely to vitamin A₁.

Some time ago also we observed that fresh rhodopsin solutions exhibit a special type of fading in darkness following exposure to light, which is absent from the same solutions after aging. We have confirmed Bliss's identification of this reaction as the conversion of retinene₁ to vitamin A₁.

The system which reduces retinene₁ is fractionated anatomically in the retinal rods. The outer segments of the rods, broken off from the underlying retinal tissue, are unable to convert their retinene₁ to vitamin A₁. In the presence of a water extract of crushed retina they do perform this conversion. On the other hand the retinal tissue from which a water extract was taken has lost this capacity. Such washed retinal tissue is reactivated by returning the washings to the solid material.

The activating effect of retinal washings on isolated outer limbs or washed retina is duplicated by a boiled muscle juice. This in turn can be replaced by reduced cozymase (reduced coenzyme I; DPN-H₂); or by a mixture of DPN and fructosediphosphate.

The conversion of retinene₁ to vitamin A₁ is therefore a reduction in which two atoms of hydrogen are transferred to retinene₁ from reduced cozymase. It is assumed that this reaction is catalyzed by an apoenzyme, retinene₁ reductase, present in the rod outer limb. This process is coupled with a second system in the outer segment which reduces DPN, using hexosediphosphate or one of its derivatives as hydrogen donor. This action of DPN brings a member of the vitamin B complex, nicotinic acid amide, into an auxiliary position in the rhodopsin system.

In the isolated retina or *in vitro* systems the reduction of retinene₁ proceeds irreversibly. Yet this reduction must be balanced by an oxidative process elsewhere in the rhodopsin cycle, since through rhodopsin as intermediate vitamin A₁ regenerates retinene₁.

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THE TONICITY-VOLUME RELATIONS FOR SYSTEMS CONTAINING HUMAN RED CELLS AND THE CHLORIDES OF MONOVALENT CATIONS

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It has been recognized since the time of the earliest investigations into the osmotic behavior of red cells that different values for volume may be found when the cells are suspended in solutions of different ionic composition but of the same depression of freezing point. The problem as to why this should occur is of historical interest because it was an observation of this type which led Moore and Roaf (Moore and Roaf, 1908; Roaf, 1912) to think of the ion distribution between the red cell and its environment as regulated by ion-binding processes rather than by permeability processes as ordinarily understood; these early observations, however, have been largely discounted, partly because there are serious technical difficulties attached to the determination of the freezing point of plasma and of hemolyzed red cells, and partly because of doubt as to the purity of the salts used and as to the reliability of the methods for measuring volume. Ege (1921) investigated the phenomenon as it presents itself when the solutions are those of salts of the monovalent anions (NaCl, KCl, KNO₃, NaSO₄, etc.). He observed differences in equilibrium volume, sometimes as great as 10 per cent, in solutions of the same depression of freezing point; he believed that these differences are best explained by assuming the rate of penetration of the different anions to be different, but was unable to account for the order in which the monovalent anions produce the anomalous effects on volume. Ponder and Saslow (1930, 1931) also noticed discrepancies in the relation between tonicity and volume when rabbit red cells are suspended in hypotonic NaCl, KCl, and LiCl, and attributed the differences to the loss of cell K being greater into some media than into others; this explanation has since been abandoned, but no other has been suggested in its place.

This paper is concerned with the tonicity-volume relations of human red cells in solutions of the salts of the monovalent cations LiCl, NaCl, KCl, RbCl, and CsCl.

1. Red Cell Fragility in Equimolar Solutions of the Chlorides of the Monovalent Cations

Table I gives the tonicity T_0 in which there is just commencing hemolysis and the tonicity T_{50} in which there is 50 per cent hemolysis in systems at 22°C. containing human red cells and hypotonic LiCl, NaCl, KCl, RbCl, and CsCl.

Description of the Hemolytic Systems.—The hemolytic systems in which T_0 and T_{50} are measured are composed of 2 ml. of a series of solutions of the chlorides of the monovalent cations, descending in tonicity from $T = 1.0$ to $T = 0.4$ by steps of $T = 0.05$, to each of which 0.5 ml. of a suspension of washed human red cells is added. The washed cells of 2.5 (0.4/ ρ) ml. of heparinized blood are finally suspended in 25 ml. of 1 per cent NaCl to make the suspension. After the addition of the red cells, the tonicities of the descending series become 1.0, 0.96, 0.92, . . . 0.44.

The chlorides of the monovalent cations, after being dried at 60°C. for several days, are freshly prepared in 0.172 M solution in water. Specimens from two sources were used: LiCl, NaCl, KCl, RbCl, and Cs from the Amend Chemical Company, and LiCl, NaCl, and CsCl prepared by Dr. Theodore Shedlovsky for conductivity work. The two sets of preparations have slightly different effects on red cell swelling and hemolysis; the swelling observed with the Amend Chemical Company preparation of

TABLE I

	T_0	T_{50}
LiCl	0.65	0.53
NaCl	0.56	0.45
KCl	0.60	0.49
RbCl	0.61	0.49
CsCl	0.59	0.47

Order, $\text{Li} > \text{K} \geq \text{Rb} > \text{Cs} > \text{Na}$.

LiCl, for example, is a little greater than that observed with Dr. Shedlovsky's preparation, and so the latter was used in the experiments of section 2. In all cases the tonicity of the 0.172 M solution was taken as $T = 1.0$, the hypotonic solutions being made by the addition of water. The pH of the solutions varied from pH 6.6 to pH 6.8 (freshly prepared solutions, glass electrode); after the addition of the cell suspension, the pH of the systems was 7.1 ± 0.05 .

The completed hemolytic systems are allowed to stand at 22°C. for 5 hours, with occasional mixing by inversion. At the end of that time the cells are thrown down, and the amount of lysis is determined from the concentration of Hb in the supernatant fluids; this is found colorimetrically, the whole procedure being very like that already described (Ponder, 1948 a).

Table I shows that the tonicities T_0 and T_{50} are not identical in their effects in the case of all the 0.172 M chlorides of the monovalent cations, and that the fragility of the cells is least in 0.172 M LiCl and greatest in 0.172 M NaCl. The order of the salts, with respect to the fragility of human red cells in them, is $\text{Li} > \text{K} \geq \text{Rb} > \text{Cs} > \text{Na}$, which is not the order of either the hydrated or the crystal radius of the ions.¹

¹ The introduction of corrections for differences in the activity coefficients makes matters worse instead of better, for the order of the activity coefficients is $\text{Li} > \text{Na} > \text{K} > \text{Rb} > \text{Cs}$, and LiCl, the strongest electrolyte, is the one which is isoplethochontic

2. The Tonicity-Volume Relations in Hypotonic NaCl and in Hypotonic LiCl Systems

The tonicity in which a red cell hemolyzes in a hypotonic system is that in which it reaches its critical volume V_h , a volume determined principally, although not exclusively, by red cell shape. When the quantity of the hypotonic medium is very great, the expression which gives the volume as a function of the tonicity T is

$$V = RW \left(\frac{1}{T} - 1 \right) + 1 \quad (1)$$

in which the initial volume V_0 of the cell is represented by unity, in which W is the water in the cell expressed as a fraction of unity, and in which R is a constant which varies from system to system. There are accordingly several ways in which variations in the fragility of a red cell, as measured by the tonicity in which it hemolyzes, can occur. The critical volume V_h itself may vary, the value of R may vary, as when there are changes from one metastable form of the red cell to another (Ponder, 1945), changes in the amount of "bound water," or when there is an escape of osmotically active substances from the cell into the hypotonic medium,² or the value of W may vary. The point which is apt to be confusing is that tonicity is classically defined in terms of the volume of the cells of the system under consideration, a solution being isotonic with plasma when it maintains the same cell volume as plasma does, *i.e.*, when it is isoplethechontic with plasma;³ we are not entitled to expect, however, that the freezing point or the activity of water will be equally depressed in all isoplethechontic solutions unless the red cell can be represented by a model of a special kind.

From a technical point of view, the analysis of the difference between the volume-tonicity relations in hypotonic NaCl and in hypotonic LiCl involves the simultaneous measurement of V_h , R , and W .

Measurement of R and W .—The red cells of human heparinized blood are washed 3 times with 0.172 M NaCl, and are then suspended (a) in 0.172 M NaCl and (b) in 0.172 M LiCl, in such proportions that the volume concentration of the cells is 0.4.

with plasma when present in the highest concentration (equimolar with 1.1 gm./100 ml. NaCl). NaCl, a weaker electrolyte, is isoplethechontic with plasma in a concentration of only 0.93 gm./100 ml.

² An escape of K from the red cell can be held responsible for a change in the value of R only if the escape is rapid, and only if it is not compensated for by an entry of an equivalent amount of Na. This point has been fully discussed elsewhere (Ponder, 1948 b).

³ This word ("volume maintaining") was introduced by Ponder and Saslow (1930) to avoid the ambiguity associated with the word "isotonic," which is often used without the realization that isosmotic solutions are not always isoplethechontic.

A series of solutions of NaCl and LiCl, varying in tonicity from $T = 1.0$ to $T = 0.4$, are prepared, a 0.172 M solution of each salt being considered, for the time being, as having a tonicity of 1.0; the hypotonic solutions are made by the addition of water. To 2 ml. of each is added 0.5 ml. of the appropriate red cell suspension (red cells suspended in isotonic NaCl being added to the series of NaCl solutions, cells suspended in isotonic LiCl being added to the series of LiCl solutions). After standing for 5 hours at 25°C., with occasional mixing by inversion, small volumes of each system are transferred to Hamburger hematocrit tubes, in which the relative cell volumes, together with the amount of hemolysis, if any, are determined (*cf.* Ponder, 1948 *c*).⁴

The expression for the volume of the red cell, regarded as an osmometer of initial volume $V_0 = 1.0$ and immersed in a limited volume of a medium of tonicity T ,⁵

$$V = \frac{RW(a - aT)}{(aT + 1)} + 1 \quad (2)$$

can be rewritten as

$$V = RW \left(\frac{1}{T + 1/a} - \frac{T}{T + 1/a} \right) + 1 = RW \cdot f(T, a) + 1 \quad (3)$$

where a is the ratio of the volume of the surrounding medium to the volume of the cell water. This expression becomes identical with expression (1) when a is infinitely great; when a has a comparatively small value, a series of values of $f(T, a)$ is calculated and used to replace values of $1/T$ (see abscissa of Fig. 1). If the red cell can be treated as an osmometer, a straight line will result when values of V are plotted against values of $f(T, a)$; this line will pass through the origin $T = 1.0, f(T, a) = 0, V = 1.0$, and its slope will be RW . The value of R can be calculated from the slope RW of the straight line when the value of W is known. It can be found by drying a small mass of red cells to constant weight at 60–80°C.

As the tonicity is reduced, a value $T_{h(0)}$ is finally reached at which the least resistant cell in the system hemolyzes. The volume which corresponds to this critical tonicity

⁴ These determinations are made by spinning with a force of $2.7 \times 10^8 G$, maintained for 30 minutes. Differences in the centrifugal force applied (and, to a minor extent, differences in the duration of spinning) result in differences in the value of R as well as in differences in the absolute lengths of the columns of packed cells. The most likely explanation for this is that the packing forces change when the cell undergoes swelling and decreases in density. A reduction of the centrifugal force to $0.7 \times 10^8 G$ results in an increase of from 0.1 to 0.15 in R . I have not been able to obtain the high values of R (average value of 0.98) found by Guest (1948) for human red cells in hypotonic NaCl, except at relatively low rates of spinning. Recently I had the experience of obtaining, in a succession of determinations, R values between 0.85 and 0.95 instead of the usual 0.7 to 0.8 (systems of human red cells in hypotonic NaCl). This was traced to the hematocrit motor needing oil.

⁵ The addition of the 0.5 ml. of suspension, which contains 0.3 ml. of NaCl or of LiCl of a tonicity of 1.0 (0.172 M), raises the tonicity of the hypotonic medium to which the cells are added, so that the final tonicities in the series are 1.0, 0.913, 0.826 . . . (common difference 0.087) instead of 1.0, 0.9, 0.8 . . . (common difference 0.1). It is the tonicity of the mixture which is denoted by T .

is $V_{h(0)}$, the critical volume for the least resistant cell in the system. If the tonicity is reduced further, there is hemolysis in the system and we are concerned with the volume of the cells which remain intact; this is $V/(1-\phi)$, where ϕ is the amount of lysis in the system ($\phi = 1.0$ for complete hemolysis). The tonicity in which $\phi = 0.5$ is the tonicity $T_{h(50)}$ with its corresponding critical volume $V_{h(50)}$ for the cell of average resistance. The true critical volumes are $V_{h(0)}/V_p$ and $V_{h(50)}/V_p$, for V_p , the volume of the cells in plasma, is not generally equal to the volume in the solution which has the tonicity denoted by 1.0 (see below).

Adjustments near the Origin.—In some types of experiment, it may be desirable to compare V_0 with a special value of V , the volume V_p of the same number of red cells in plasma. If $V_0 = V_p$, the salt solution of tonicity $T = 1.0$ is isotonic (isoplethechontic) with plasma. If the salt solution of $T = 1.0$ is not isoplethechontic with plasma, V_0 will equal $V_p + \Delta W$, and ΔW , which can be either positive or negative, will be the amount of water which enters (or leaves) the cell when it is transferred from plasma to the solution for which T has been put equal to 1.0. There will be a point on the linear tonicity-volume relation which has coordinates V_p and a special value of $f(T, a)$; from this special value can be calculated a value of T which can be used as a number by which the concentration of the salt solution under consideration must be multiplied in order to give a salt solution isotonic with plasma.

When the salt solution of $T = 1.0$ is not isoplethechontic with plasma, two consequences follow upon the taking of $T = 1.0$ as the origin of the coordinates of the tonicity-volume relation. The first is that red cells immersed in the solution of $T = 1.0$ (supposedly isoplethechontic with plasma, but not really so) will gain (or lose) water; this will change the slope of the line relating V and $f(T, a)$ from RW to RW_1 .⁶ If V_p is less than V_0 , the cell will appear to behave as a better osmometer than it really is; alternatively, if V_p is greater than V_0 , the cell will appear to behave as a poorer osmometer than it really is. The second is that if the cells begin to hemolyze at a critical volume V_h , and if the ratio V_h/V_0 is calculated from the value of V_0 found in the solution of tonicity 1.0 (not isoplethechontic with plasma) instead of from V_h/V_p , the critical volume will appear to be spuriously small in relation to the initial cell volume if V_p is less than V_0 and spuriously great if V_p is larger than V_0 . Serious discrepancies can be introduced by an error of this kind.⁷

⁶ $W_1 = (W \pm \Delta V_0)/(V_0 \pm \Delta V_0)$.

⁷ It is not the purpose of this paper to discuss the concentrations of NaCl and of other salts which have been found to be isoplethechontic with normal human plasma collected under oil. This aspect of the problem has been dealt with by Christensen and Warburg (1928) and by Kirk, Sorensen, Trier, and Warburg (1941); the concentration of NaCl which they found to be isoplethechontic with plasma is about 0.150 M. Other values found, usually with fewer precautions as regards preventing the escape of CO₂, vary from 0.145 M to 0.190 M. The pH of NaCl solutions is generally less than that of plasma and particularly of plasma which has been exposed to air, and so it is to be expected that the cation content of an NaCl solution isoplethechontic with plasma would be somewhat greater than that of plasma itself. The point which this paper emphasizes is that the cation content of a LiCl solution isoplethechontic with plasma would be greater still, the difference between the concentrations of NaCl and of LiCl not being attributable to differences in pH.

Fig. 1 shows the results obtained with NaCl and with LiCl. The points for the NaCl systems lie on a straight line passing through the origin with a slope $RW = 0.50$; assuming W to be 0.7, $R = 0.72$. The tonicity in which the cells have the same volume V_p as they have in plasma is 0.93 gm./100 ml., and in the tonicity $T = 1.0$ (1.0 gm./100 ml.) the cells have decreased in volume from 1.04 to 1.0 units of volume. Allowance for this loss of water would increase the value of R from 0.72 to 0.75. At the upper end of the line, $p = 0.0$ when $V = 1.35$; remembering that the cell in plasma has a volume of 1.04 on the scale in use, the critical volume for the cell of least resistance is $V_{h(0)} = 1.30 V_p$.

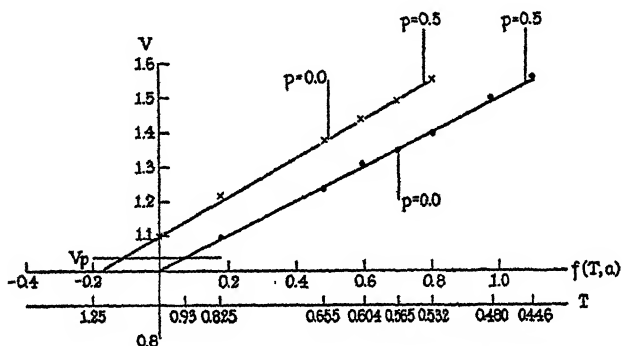


FIG. 1. Volume-tonicity relations for systems containing NaCl (filled circles) and LiCl (crosses). Ordinate, cell volume; abscissa, tonicity T and its function $f(T, a)$. Commencing hemolysis at $p = 0.0$; 50 per cent hemolysis at $p = 0.5$. The volume of the cells in plasma is V_p .

The points for the LiCl systems lie on a straight line passing through the point ($T = 1.0$, $V = 1.10$) and having a slope $RW = 0.56$; assuming W to be 0.7, $R = 0.80$. The tonicity in which the cells have the same volume V_p as they have in plasma is equimolar with 1.1 gm./100 ml. NaCl, and in a tonicity of $T = 1.0$ the cells have increased from 1.04 to 1.10 units of volume. Allowance for this entry of water reduces the value of R from 0.80 to 0.78, which is not significantly greater than the corrected value of R , 0.75, for the NaCl system. At the upper end of the line, $p = 0.0$ when $V = 1.38$, and so the critical volume for the cells of average resistance is almost the same as that found in the NaCl system.⁸

⁸ The volumes at which $p = 0.5$ in the two systems may also be compared. They are $1.68 V_p$ in each case. Once the cells begin to hemolyze, however, the relation of $V/(1-p)$ to tonicity becomes dependent on factors which are not yet sufficiently defined. Often the relation continues along the same straight line (as in the experiment illustrated in Fig. 1) until p is 0.7 or even 0.8; after this the measurements be-

The difference between the tonicity-volume relations found in NaCl and in LiCl respectively is accordingly almost entirely accounted for by the concentrations of isotonic (isoplethochontic with plasma) solutions of NaCl and of LiCl differing by about 18 per cent. This is essentially the same result as Ege obtained with his series of monovalent anions, and it does not seem possible at present to state it in any simpler form. Equimolar concentrations of the chlorides of the monovalent cations unquestionably have specific effects on the volume which red cells attain when immersed in them. One would expect these specific effects to be related to properties such as the hydration of the cations, but this explanation would be tenable only if additional specific properties were ascribed to K and Na, so as to make the effect of K equal to that of Rb and to move Na to the end of the series. It is true that K and Na are special ions so far as the red cell is concerned, since they are normal constituents, but one cannot have much confidence in a type of explanation which allows of two exceptions in a series of five.

It can be shown that the effect of equimolar concentrations of NaCl and LiCl on red cell volume is reversible; there are also small differences in the rate at which K is lost by red cells into solutions of the two salts.

Reversibility of the Effect.—This can be demonstrated by preparing suspensions of identical numbers of cells in (1) 1.0 gm./100 ml. NaCl, (2) 0.72 gm./100 ml. LiCl, and (3) suspended in 0.72 gm./100 ml. LiCl for an hour, then washed with 1.0 gm./100 ml. NaCl, and finally suspended in the latter medium. Identical suspensions are most easily made by weighing 2 gm. of a red cell suspension of volume concentration $\rho = 0.4$ (in 1.0 gm./100 ml. NaCl) into three weighed tubes. The cells in the three tubes are thrown down and the supernatant fluids are removed; 1.0 gm./100 ml. NaCl is then added to the first tube, and 0.72 gm./100 ml. LiCl to the second, until the weight of the contents of each tube is 2 gm. About 10 ml. of 0.72 gm./100 ml. LiCl is added to the third tube, the contents of which are allowed to stand for about an hour; the cells are thrown down, washed once with 1.0 gm./100 ml. NaCl, thrown down again, and then made up to a weight of 2 gm. with 1.0 gm./100 ml. NaCl. The volume concentrations of the cell suspensions in the three tubes are found by spinning at $2.7 \times 10^5 G$ for 30 minutes. They will be found to be in the ratio 1.00, 1.10, and 1.00, which shows that the effect of 0.72 gm./100 ml. LiCl on volume is reversible by washing with equimolar NaCl.

K Losses.—The full effect of LiCl on red cell volume can be observed if the cell volume is measured, by the hematocrit, as soon after the addition of the cells to 0.172 M LiCl as the hematocrit method allows one to measure it, and allowing the cells to

come unreliable. Sometimes the values of $V/(1-p)$ corresponding to $p = 0.5$ and more are unexpectedly great and lie on a curve concave to the $f(T, a)$ axis instead of on a continuation of the straight line. In still other systems, the values of $V/(1-p)$ go through a maximum when p has a value in the neighborhood of 0.5 (Ponder, 1948 c). The cause of these troublesome variations in the apparent volume-tonicity relations of the intact cells requires further study.

stand for periods up to 5 hours in 0.172 M LiCl does not increase the magnitude of the effect appreciably. It is accordingly not surprising to find that the losses of K which take place into isotonic or hypotonic LiCl are so small and take place so slowly that they cannot be held responsible for the phenomena under discussion. At 25°C., K losses amounting to 0.08, 0.08, 0.05, and 0.06 when expressed as fractions of the initial cell K are observed in the first 24 hours into 0.172 M NaCl, 0.172 M LiCl, hypotonic NaCl ($T = 0.6$), and hypotonic LiCl ($T = 0.6$), respectively.

SUMMARY

1. Differences in the fragility of human red cells are observed in equimolar solutions of the chlorides of the monovalent cations. The cells are most fragile in LiCl and least fragile in NaCl, the salts falling in the order $\text{Li} > \text{K} \geq \text{Rb} > \text{Cs} > \text{Na}$.

2. The difference between the tonicity-volume relations in systems containing LiCl and systems containing NaCl lies in the molarity of the solution of LiCl which is isotonic (isoplethochontic) with plasma being considerably greater (0.189 M) than the molarity of the solution of NaCl which is isotonic (isoplethochontic) with plasma (0.160 M). The difference cannot be stated meantime in any simpler terms than these; if the activity coefficients are taken into account, it becomes even greater. The tonicity-volume relations for the two systems are otherwise almost identical; the value of R for the two systems is almost the same, the critical volumes at which the cells of least resistance hemolyze are almost identical, and the critical volumes at which the cells of average resistance hemolyze are almost identical.

3. The LiCl effect on volume occurs as soon after the addition of the cells to 0.172 M LiCl as the hematocrit method allows one to measure it. It is reversible by washing with 0.172 M NaCl.

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THE TONICITY-VOLUME RELATIONS FOR HUMAN RED CELLS SUBJECTED TO THE ACTION OF HEAT, WITH SPECIAL REFERENCE TO PROLYTIC K LOSS

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In the course of their investigations on the effects of thermal injury to red cells, Ham, Shen, Fleming, and Castle (1948) have observed that the extent to which swelling occurs in hypotonic media of different tonicity is approximately the same for unheated red cells and for cells heated for short periods to temperatures between 49.6° and 50.6°C. Their conclusion that these temperatures have only a small effect on the permeability of the red cell membrane and on the osmotic activity of their contents is interesting because of its implications, since the heated cells show fragmentation and since cells exposed to temperatures a few degrees higher show even greater fragmentation accompanied by the appearance of innumerable tiny particles similar to the threads and droplets observed during the disintegration of ghosts (Furchgott, 1940). It is not likely that a simple bag-like structure, in which a fluid interior is enclosed in an investing membrane, could undergo such extensive fragmentation without alteration in its osmotic properties, and so the observations on the effect of heating may be expected to have a bearing on the problem of red cell structure; it is with this aspect of the investigation that this paper is concerned.

Preliminary experiments having shown that the osmotic behavior of the red cell undergoes a fundamental change when it is heated for 2 minutes to a temperature in the neighborhood of 50°C., the results will be described by considering what occurs in two systems, the first containing human red cells heated for 2 minutes at 48°C., and the second containing human red cells heated for 2 minutes at 52°C.

1. Heating for 2 Minutes at 48°C.

The cells of about 15 ml. of heparinized human blood are washed several times in 1.0 per cent NaCl, and are then suspended in sufficient 1.0 per cent NaCl to give a volume concentration of $\rho = 0.4$. This suspension is divided into two equal parts, one of which is to be heated while the other is not heated. The heating is carried out in very much the same way as that described by Ham, Shen, Fleming, and Castle, a small volume of the cell suspension being immersed in a large volume of water heated to a little above the desired temperature. The time taken for the suspension to reach the temperature of the

surrounding water was 1.5 minutes; after the period of heating, the suspension was allowed to cool to 25°C.

A series of NaCl solutions of decreasing tonicity ranging from $T = 1.0$ to $T = 0.4$ is prepared by adding water to 1 per cent NaCl, and 0.5 ml. of the unheated suspension is added to 2 ml. of each of the solutions of the series; similarly, 0.5 ml. of the heated suspension is added to 2 ml. of each of the solutions of the series. The addition of the 0.5 ml. of suspension, 0.3 ml. of which is 1 per cent NaCl, raises the tonicity of the hypotonic solutions, so that the final tonicities in the series are 1.0, 0.913, 0.826 . . . (common difference 0.087) instead of 1.0, 0.9, 0.8 . . . (common difference 0.1). The contents of the tubes are kept at 25°C. and are mixed by occasional inversion. At the end of 1 hour, samples are removed from each system for the determination of red cell volume and of the extent of hemolysis. The volumes are found by spinning in Hamburger hematocrit tubes at $2.7 \times 10^8 G$ for 30 minutes, and the amount of hemolysis is found from colorimetric determination of the Hb in the supernatant fluids of the systems. The volume occupied by the intact red cells of any system is $V/(1 - p)$, V being the volume as read off in the hematocrit tube.

The volumes occupied by the intact red cells are expressed as a fraction of V_0 , the volume in 1 per cent NaCl, and are then plotted against the function of T , $f(T, a)$, which applies to the system under consideration (see expression 3, Ponder, 1949).¹ If the cell behaves as an osmometer, the result is a straight line passing through the origin. The properties of this line have already been discussed (Ponder, 1949). Its slope is RW , where W is the amount of cell water expressed as a fraction of the initial volume of the cell, and where R is a constant which measures the degree of perfection of the osmometer. W can be found independently by drying to constant weight; in these experiments it has been taken as 0.7 throughout. As one proceeds upwards along the line towards larger volumes and lower tonicities, a tonicity is reached at which lysis of the least resistant cell occurs; this is the critical volume $V_{h(0)}$. Proceeding still further along the line in an upward direction, tonicities are reached at which the cell of average resistance, and finally the cell of greatest resistance, hemolyzes; these are the critical volumes $V_{h(50)}$ and $V_{h(100)}$.

The results obtained in an experiment in which the osmotic behavior of untreated red cells is compared with that of red cells which have been heated for 2 minutes at 48°C. are shown in Fig. 1. Unheated and heated red cells seem to be equally good osmometers,² the points lying satisfactorily along the

¹ This is nearly the same as plotting V against $1/T$. It is permissible, indeed, to plot V against $1/T$ in all except the final computations.

² Ham, Shen, Fleming, and Castle have observed that unheated cells and cells heated to between 49.6° and 50.6°C. behave as equally good osmometers (their Table 4), but their value of R is very low (0.4). Their values of T seem to need some

same straight line (marked *A* in the figure), with a slope $RW = 0.51$; assuming $W = 0.7$, $R = 0.73$. The one difference between the unheated and the heated red cells is that the latter begin to hemolyze at a tonicity in the neighborhood of 0.65 while the former do not begin to hemolyze until the tonicity is about 0.57. The explanation put forward by Ham, Shen, Fleming, and Castle to account for this difference is that the critical volume for hemolysis is smaller in systems containing heated red cells because the cells fragment and become more spherical in doing so.

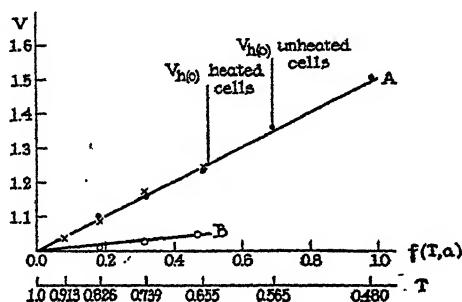


FIG. 1. Tonicity-volume relation for unheated and for heated red cells. Ordinate, cell volume; abscissa, tonicity T and its function $f(T, a)$. Filled circles, unheated cells; crosses, cells heated to 48°C. for 2 minutes; open circles, cells heated to 52°C. for 2 minutes.

Ham, Shen, Fleming, and Castle's hypothesis as to the effects of fragmentation on critical volume can be put into a more quantitative form in the following way. Let us divide a big sphere of volume V into 2, 4, 8, . . . N equal spheres;³ the radius of each little sphere will then be

$$r = \sqrt[3]{\frac{3V}{4\pi N}}$$

and its area will be $4\pi r^2$. The total area of all the little spheres resulting from the subdivision of the big sphere will be

$$A = 4\pi N \left(\frac{3V}{4\pi N} \right)^{\frac{2}{3}} \quad (4)^*$$

* The equations for this paper and the preceding one are numbered consecutively.

numerical correction, if for no other reason than that 0.85 per cent NaCl is not an isoplethochontic solution for human red cells.

³ Division of a sphere (or disk) into two *equal* spheres gives a *maximum* increase in the surface/volume ration. The real sequence of events is that the disk divides into two or more fragments, not usually equal in size, and that these round up to become spherical.

The hypothesis under consideration depends on the fundamental idea that lysis in a hypotonic system occurs when the surface A of the swollen and spherical cell of volume V , or the surface A of N swollen and spherical fragments of total volume V , equals the initial surface area A_0 of the discoidal cell of initial volume V_0 . For the human red cell, $A_0 = 163 \mu^2$, and so we can insert various values of N in expression (4) and compute the value of V which will give $A = 163$. The results are tabulated in the first two columns of Table I, the value for $V = V_0 = 87 \mu^3$ being obtained by interpolation.⁴

The approximate tonicities in which these volumes occur can be computed from expression (1), some value for R being assumed. The last two columns of Table I give these values of T on the assumption that $R = 1.0$ and that $R = 0.75$ (a frequently occurring value) respectively. Other things being equal, a cell which is a perfect osmometer and which can remain intact in a tonicity of 0.365 will hemolyze in a tonic-

TABLE I

N	V	T , $R = 1.0$	T , $R = 0.75$
1	193	0.365	0.303
2	136	0.545	0.480
4	98	0.875	0.845
5.3	$87 = V_0$	0.100	1.000
8	69	1.430	1.640

ity of 0.545 if, after swelling, it divides into two equal spheres, and if divided into 5.3 equal spheres it will hemolyze, without any swelling at all, in a tonicity of 1.0 because the total surface of the 5.3 spheres equals $163 \mu^2$, the area of the original disk.

This hypothesis can be tested if N is determined by counting the number of intact cells plus fragments present in the suspension before and after heating;⁵ N is the ratio of the second number to the first, and is the average number of fragments into which a single cell divides; some cells of the system will no doubt divide into fewer fragments

⁴ In systems containing unheated red cells, lysis begins in a tonicity of 0.566 and there is 50 per cent lysis in a tonicity of 0.448 (Ponder, 1948 *a*). On the line marked A in Fig. 1, the tonicity of 0.448 corresponds to a volume V/V_0 of 1.57, which is considerably less than the value of $V/V_0 = 193/87 = 2.22$, derived from the values (Table I) on the assumption that $A_0 = 163 \mu^2$. It may be that A_0 is somewhat overestimated, but it is more likely that the average cell hemolyzes when its volume is substantially less than that of the sphere of area A_0 (Ponder and Robinson, 1934).

⁵ The number of cells plus fragments is found by counting on a hemocytometer chamber under quite high illumination. When there is extensive fragmentation, the cell suspension is diluted to a greater extent than when there is not. Care must be taken to allow time for the smaller particles to settle on to the floor of the chamber. Provided that the degree of fragmentation is relatively small, the precision with which N can be determined is greater than one might expect. It should be noticed that ghosts are not counted.

and some into more. The division into the N fragments of Table I is therefore associated with 50 per cent hemolysis ($p = 0.5$) in the tabulated tonicities.

In the case of systems containing red cells heated to 48°C. for 2 minutes, the amount of fragmentation is usually very small ($N = 1.01$ to 1.05) and so it cannot be decided with certainty whether it is sufficient to account for the increase in the lysis observed in hypotonic media. The fragmentation can be increased, however, by heating the cells to 48°C. for longer times, *e.g.* for 4 minutes or for 8 minutes; heating for 4 minutes usually gives $N = 1.3$ to 1.5 , and heating for 8 minutes gives $N = 1.6$ to 1.8 .⁶ Fragmentation to the extent observed after 8 minutes' heating would certainly account for the difference in the tonicities at which unheated cells and heated cells hemolyze, but the impression which one gets from experiments in which increasing values of N are obtained by heating for increasing times at 48°C. is that the effect on fragility is out of proportion, in the direction of being too great, to the fragmentation as measured by N . This point will be referred to again later.

The tonicity-volume relation is substantially the same for red cells which have been heated to 48°C. for 2, 4, and 8 minutes; *i.e.*, the experimental points lie along the same straight line (such as that marked *A* in Fig. 1) as do those for unheated red cells. The only difference lies in the tonicity at which lysis begins; this becomes higher as the duration of the heating is increased, as would be expected from the increase in the amount of fragmentation. Since the associated values of N vary from 1.0 to 1.6, red cells from which fragments have broken off must have the same tonicity-volume relation as unfragmented ones have, and must behave as osmometers of the same degree of perfection ($R = 0.65$ to 0.75 in different experiments).⁷ This means that the properties which are usually referred to as the osmotic properties of the red cell are not necessarily dependent on the integrity of the cell as a unit.

⁶ Considerable individual variation is met with as regards the amount of fragmentation which occurs at temperatures between 48° and 52°C., and the value of N has to be found by trial in the case of each sample of red cells. Appreciable variations in the fragmentability at a given temperature, *e.g.*, 50°C., occur when the red cells are abnormal, as in some of the anemias. The temperature to which the cells are heated seems to be more important than the duration of heating in determining the amount of fragmentation. The amount of fragmentation also depends, to a minor extent, on the vigor with which the heated system is stirred.

⁷ Superficially, it would also seem obvious that the small fragments must have the same tonicity-volume relation as the intact red cells have. The contribution which the fragments make to the total volume, however, is only $v(N - 1)/N$, where v is the ratio of the volume of the average fragment to the volume of the intact cell. The volume of the average fragment may be quite small, and so the contribution to the total volume may be so small (3 per cent or less) that a complete failure of the fragments to swell would not affect the experimental tonicity-volume relation appreciably.

2. Heating for 2 Minutes at 52°C.

Quite a different result is obtained if the red cells are heated to 52°C. for 2 minutes. Much more fragmentation takes place, and since a fraction p of the cells are hemolyzed, $N/(1 - p)$ is used as an approximate measure of the extent of fragmentation instead of N itself. In these systems, $N/(1 - p)$ varies between 3.0 and 4.5, and the tonicity-volume relation for the intact cells⁸ is now represented by the straight line marked *B* in Fig. 1. The slope RW of this line is 1.0, whence $R = 0.14$, a value much smaller than that which applies to the straight line for the unheated cells or for cells heated to 48°C. for 2 minutes (line marked *A* in Fig. 1). It is clear that the ability of the cell to swell in hypotonic media is greatly impaired after heating to 52°C. for 2 minutes; the systems, moreover, show much more hemolysis than that which occurs in systems containing unheated cells. The latter do not begin to

TABLE II

	<i>T</i>	<i>p</i>	K	Na	K + Na
			<i>m.eq./l.</i>	<i>m.eq./l.</i>	<i>m.eq./l.</i>
Unheated	1.0	0.0	100	39	139
Heated, 52°C.	1.0	0.2	89	48	137
“ “	0.78	0.6	56	56	105
“ “	0.65	0.7	34	62	96

hemolyze until the tonicity falls to about 0.55, at which tonicity $V_{h(0)} = 1.44$; the former show about 20 per cent lysis even in $T = 1.0$, whereas in the tonicities $T = 0.8$ and 0.6 , there is 60 and 70 per cent hemolysis respectively.

Of the many possible explanations for the low value of R found in systems containing red cells heated to 52°C. for 2 minutes, the one which is most readily investigated is that the prolytic K-Na ion exchange is of such a magnitude as to enable the cells to come into equilibrium with a hypotonic environment without undergoing any large volume change. Measurements of the K and Na contents of unheated and of heated red cells, added to systems of tonicity 1.0, 0.75, and 0.6 were carried out as described by Ponder (1947) the intact cells being separated as quickly as possible after addition to the saline media. The separation and packing, carried out in a high speed centrifuge, took about 15 minutes, and it ought to be pointed out that the material separated con-

⁸ The volume of the intact cells is taken as being $V/(1 - p)$, but this is only an approximation because the loss of Hb from the cells of these systems is not all-or-none. It is not difficult to distinguish in the hematocrit tube between the column of intact cells and the grey column of ghosts above it. The intact cells certainly have ghosts mixed in with them, but the contribution of the ghosts to the total volume is probably quite small.

tained red cells in all stages of fragmentation together with a small proportion of ghosts. Representative results are given in Table II.

Heating to 52°C. for 2 minutes is followed by a considerable K-Na exchange (11 per cent K lost and an equivalent amount of Na gained) even when the cells are placed in a medium of $T = 1.0$; when the cells are added to hypotonic media, the K losses and the Na gains are much greater (66 m.eq./liter, or 66 per cent, of cell K lost in a system of tonicity 0.65; 23 m.eq./liter of Na gained). In each case, the sum $K + Na$ is approximately that which would provide an equilibrium with the medium surrounding the cells.

At temperatures higher than 52°C. the loss of K from the intact cells of the system is still greater. The K loss at any temperature can be calculated from measurements of the amount of hemolysis p in the system together with measurements of the K lost into the suspension medium, expressed as K_p , a fraction

TABLE III

Temperature °C.	$N/(1-p)$	$T = 1.4$			$T = 1.0$			$T = 0.8$		
		p	K_p	F	p	K_p	F	p	K_p	F
46	1.05	0	3	0.03	0	3	0.03	0	4	0.03
49	1.8	1	9	0.08	2	9	0.08	3	10	0.08
51	4.2	13	26	0.15	27	44	0.23	55	72	0.38
54	4.3*	33	45	0.18	39	55	0.26	61	81	0.51

* Does not include many uncountable myelin forms.

of the total K obtainable from lysis of all the cells. The fraction of its K which the intact cell loses is given by

$$F = (K_p - p)/(1 - p)$$

and the extent of fragmentation in the preparation is measured by $N/(1 - p)$. Table III gives values for N , p , and F obtained for systems in which red cells, heated to various temperatures for 2 minutes, are added to 1.4, 1.0, and 0.8 per cent NaCl respectively.

The fraction of its initial K which the average intact red cell loses (F) increases with increase in the temperature to which the cells are heated and with decrease in the tonicity T . The K losses shown in Table III occur within the 2 to 3 minutes necessary for the separation of the cells from the supernatant fluids of the systems, and are probably great enough⁹ to account for the small values of R found for cells heated to temperatures of 52°C., and temperatures

⁹ To account completely for the small value of R , the losses of K would have to take place almost immediately after the addition of the heated cells to the hypotonic media. This point has been fully discussed elsewhere (Ponder, 1948 b).

above this, for 2 minutes. At the same time the amount of hemolysis p and the fragmentation $N/(1 - p)$ increase with temperature and with decrease in the tonicity of the system. The increase in the amount of hemolysis in tonicities between 1.0 to 1.4 would be easily accounted for on simple geometrical grounds if the cells which hemolyze could be identified as the fragmented ones, for when the average value of $N/(1 - p)$ for the system is 4.2, some cells would certainly have divided into 5.3 or more fragments. This, however, is not the whole explanation, for examination of the intact cells and of the ghosts in such a system shows that both large (probably unfragmented) cells and small fragments remain intact, and that the system contains the ghosts of both large cells and small fragments. Fragmentation, with its effects on the surface/volume ratio, is accordingly not the only factor involved in the hemolysis. The other factor likely to enter into the situation is the effect of heat on the cohesion of the cell surface ultrastructure and perhaps of internal ultrastructures as well.¹⁰ A process of disintegration added to one of fragmentation would account for all the results obtained at the higher temperatures, and would be expected to result in the prolytic loss of K, the appearance of the "polymorphic fragments and innumerable minute particles," and in the loss of Hb in the amounts observed.¹¹

SUMMARY

1. The volume-tonicity relations for human red cells exposed to a temperature of 48°C. for 2 minutes remain the same as those for unheated human red cells. The heated systems show lysis in higher tonicities than the unheated systems do; this is probably largely due to fragmentation with its effect on the geometry of the situation, as suggested by Ham, Shen, Fleming and Castle. When the cells are heated to 48°C. for longer times, the amount of fragmentation becomes considerable, but the volume-tonicity relation remains the same as before; the properties which are usually referred to as the osmotic properties of the red cell are accordingly not necessarily dependent on the integrity of the cell as a unit.

2. Heating to 52°C. for 2 minutes profoundly modifies the volume-tonicity relation, very little swelling now occurring even in tonicities as low as 0.6.

¹⁰ This possibility has been mentioned by Ham, Shen, Fleming, and Castle (a "decrease in the inherent strength of the cell envelope").

¹¹ Heating chicken red cells to between 50° and 55°C. for 30 to 60 minutes results in a 70 to 80 per cent decrease in the rate of O₂ consumption, a reduction of more than 50 per cent in anaerobic glycolysis, and a decrease of about 40 per cent in the lipid content of the cells (Hunter and Stringer, 1943). Very little cholesterol is lost from the human red cell during the much shorter periods of heating employed in the experiments of this investigation. The effect of longer periods of heating on the loss of cholesterol and of other lipids requires investigation.

This is partly accounted for by the large K losses and K-Na exchanges which occur and which become greater as the tonicity is reduced and as the temperature is increased. Fragmentation and hemolysis also increase, the latter out of proportion to the expected effects of the former. Direct effects of heat on the cohesion of the red cell ultrastructure are probably involved.

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APPENDIX

Like the properties which are usually referred to as the osmotic properties of the red cell, the properties which determine its resistance to the lytic effect of substances such as saponin and digitonin are not necessarily dependent on the integrity of the cell as a unit. This can be shown by heating washed human red cells in saline to temperatures which produce fragmentation ($N = 1.8$ to 3.0), and then comparing the resistance to lysins of the cells plus fragments with that of unheated cells.

TABLE IV

	Saponin			Digitonin	
	Unheated	Heated		Unheated	Heated
	$N = 1.0$	$N = 1.1$	$N = 2.8^*$	$N = 1.0$	$N = 2.8^*$
Median	11.2	12.5	17.0	8.0	10.6
Lower extreme	4.0	4.2	4.9	5.0	6.0
Upper extreme	70.0	83.0	95.0	12.0	35.0

Determinations at end of 5 hours at 30°C. and pH 6.5.

* Spontaneous hemolysis in these systems, 23 per cent.

Since it is possible that fragmentation would produce changes in the distribution of the resistances of the cells to a lysin, the form of the whole resistance distribution should be determined. The method for doing this has already been described (Ponder, 1948 *a*). Using saponin and digitonin as the lysins, the characteristics of the frequency distribution (position of median and of both extremes) are tabulated in Table IV for various values of N .

In the case of both lysins, the heated cells appear *more* resistant. There is no evidence of the polymodalities which would suggest that intact cells and fragments have a different resistance. Many fine particles, resembling myelin forms, are found in the saponin systems in which there is complete hemolysis of the heated red cells; the cloud seen in the systems containing the higher concentrations of digitonin, on the other hand, contains a mixture of cells and Hb-bearing particles of various sizes.

The greater resistance observed in the systems containing heated cells is attributable to the supernatant fluid derived from a suspension of red cells heated for 2 minutes to 50°C. containing sufficient inhibitors, both for saponin and for digitonin, to account for the differences shown in Table IV. An antisphering factor is one of these inhibitors; cholesterol in small amounts may be another (see footnote 11). When the effect of these inhibitors is allowed for, little, if any, difference is left to be accounted for by fragmentation, and the conclusion is that fragmented cells and the particles derived from them have substantially the same resistance to these lysins as the intact cells have; *i.e.*, that the resistance to lysis by saponin and digitonin does not depend on the integrity of the cell as a unit. This conclusion can be verified by the direct microscopical observation that cells and Hb-bearing fragments are hemolyzed by saponin and digitonin regardless of their size.

ON THE ORIGINS OF DORSAL ROOT POTENTIALS*

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Observations of potential changes occurring in dorsal roots during activity of the spinal cord date from the experiments of Gotch and Horsley, commenced in 1888 and fully described in their Croonian Lecture of 1891 (18). Recent attempts at analysis and interpretation of such potential changes may be said to begin with the work of Barron and Matthews (1), who described a simple fluctuation of long duration, provoked by afferent stimulation and having negative electrical sign at the more centrally located of two leads placed on the central segment of an isolated severed dorsal root: the so called dorsal root potential. So far as this single potential change is concerned the observations of Barron and Matthews are substantially correct. During the past decade essential experimental confirmation has come from the work of Bonnet and Bremer (2, 3), Bremer and Bonnet (5), Bremer, Bonnet, and Moldaver (6), Dun and Feng (11), Eccles and Malcolm (16), and during the course of the present investigation.

In contrast to the general agreement hitherto found at the level of observation, there has been widespread divergence of opinion concerning interpretation, due in part to the lack of an adequate theoretical background and in part to the lack of an adequate description of the sequence of potential changes that constitute the dorsal root potential. The present account of dorsal root potentials in the main is concerned with those parts of the sequence that have not received due attention; it adds little to what is already known about that part of the sequence represented by the negative deflection of Barron and Matthews.

Procedure

Bullfrog and cat preparations have been employed. In the former entire dorsal roots were isolated for stimulation and recording, but in the latter it was necessary to segregate individual rootlets to obtain well defined root-cord junctions. The roots or rootlets, severed distally, were raised into an insulating medium (paraffin) and fitted with electrodes as desired. Recording leads routinely were placed on dorsal roots one close to, but not touching, the spinal cord, the other at a distance toward the severed end. Throughout the present discussion these will be designated the proximal

* Presented at the Atlantic City meeting of the American Physiological Society (21).

and distal leads respectively, and the sign of a potential change, as is customary in work with dorsal root potentials, will be expressed in reference to the proximal lead. When stimulating electrodes were applied to roots from which recordings were to be made they were placed near the severed end distal to the distal recording lead. Any root that was stimulated to carry an afferent volley will be designated the active root, others will be designated neighboring roots. Direct coupled amplification has been standard practice.

The Problem

Fig. 1 illustrates potential changes in bullfrog dorsal roots evoked by single dorsal root volleys and recorded, in A, from the active root and, in B, from a

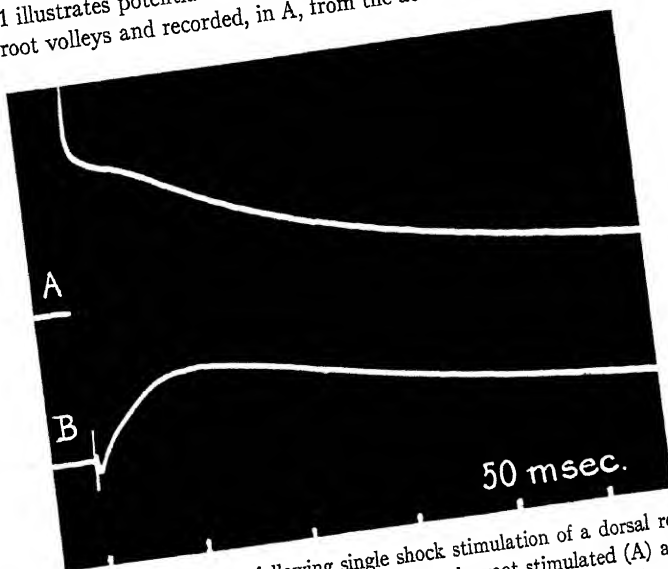


FIG. 1. Potential changes following single shock stimulation of a dorsal root and recorded by means of an electrode pair applied to the root stimulated (A) and to a neighboring root (B). Negativity at the root lead closer to the cord, in this and all other figures, is recorded upwards. Bullfrog preparation.

neighboring root of the same side. In record A, the tail of the large diphasic (positive-negative) spike potential of the traveling afferent volley may be seen, followed by a prolonged negative potential. In record B there is a sequence of potential changes culminating in a prolonged negative potential, that is the dorsal root potential of prior description. During the first 50 msec. following stimulation the relation between potential changes in the active and neighboring roots is not immediately apparent, but beyond that interval the changes in the two roots are comparable in size, duration, and electrical sign.

One must assume that the recording from an active root is of complex origin. Contributing to the over-all potential change will be: (a) potential gradients instituted in the dorsal root fibers by the stimulating current, a not insignifi-

cant factor considering the necessary proximity of electrodes placed four on a root, (b) after-potentials of the root fibers consequent upon conduction of a volley of impulses, a factor recognized by Woolsey and Larrabee (28), (c) gradients that might exist in the intramedullary segments of the stimulated fibers and which could propagate themselves back into the extramedullary segments, and (d) electrotonic potentials appearing in the root as the result of polarization of its intramedullary projection by the flow of current about active neurons.

Dorsal root potentials recorded in a neighboring root are less complex in origin than those of the active root, for the direct consequences of the flow of stimulating current and of impulse conduction cannot be contributing fac-

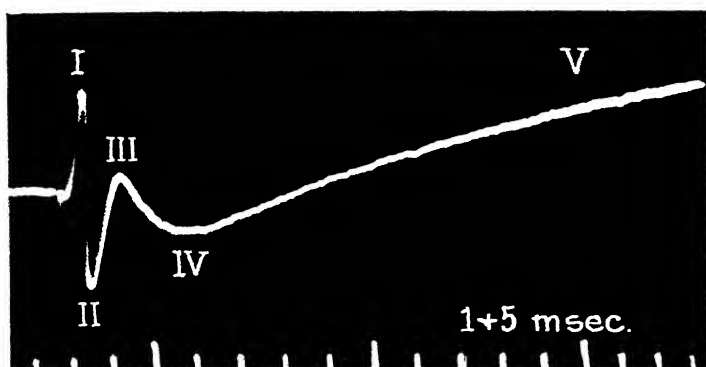


FIG. 2. Early course of dorsal root potential recorded, as in Fig. 1 B, from a root adjacent to that stimulated to show in greater detail the succession of deflections, D.R.I, II, III, IV and the beginning of D.R.V, which last is the dorsal root potential of prior description. Bullfrog preparation.

tors. A glance at record B of Fig. 1 reveals that there exist potential changes in a neighboring root additional to and anteceding the negative wave of the usual descriptions. Although these potential changes are to be found partially resolved in many of the previously published records of dorsal root potentials, they have been neglected except by Eccles and Malcolm (16) who regard them as artifacts introduced by contact between proximal recording lead and spinal cord. Such is not the case in the present recordings for which contact between lead and cord was scrupulously avoided. Furthermore the characteristic sequence is still recorded, but with the anticipated decrement, after the proximal lead is moved distally along the dorsal root.

Recorded by the use of higher oscillograph sweep speed, the early course of the dorsal root potential in a neighboring root may be seen in greater detail. Fig. 2, presenting such a recording, reveals that there are in all five deflections in the potential sequence. These deflections in order of temporal sequence are

to be designated D.R.I, D.R.II, D.R.III, D.R.IV, and D.R.V. Of these D.R.V is the negative wave of Barron and Matthews.

The dorsal root potential of a neighboring root is propagated electrotonically to the extramedullary segment of the contained primary afferent fibers from their intramedullary projections. The spinal cord constitutes a volume conductor within which the intramedullary projections of primary afferent fibers may be polarized by physical spread of current flow about active nervous elements. Such polarizations are the cause of electronic propagation into the extramedullary segment. Since the dorsal roots are raised into an insulating medium that extends to the cord junction, there is no significant *physical* spread of current from the cord into the extramedullary segment.

As a convenient simplification all the neurons within the cord are considered together as secondary neurons. This being done, one may state that polarization of the intramedullary segment must be due to either (*a*) the activity of primary afferent fibers or (*b*) secondary neurons. Now it is immediately obvious that D.R.I, II, and III together bear some relation to the triphasic intramedullary spike potential (17) of a primary afferent volley occupying the intramedullary segment of the stimulated dorsal root fibers. For this reason their origin may be ascribed with confidence to activity of primary afferent fibers. Secondary neurons clearly are responsible for D.R.V, as was first suggested by Bonnet and Bremer (2). Concerning the general properties of D.R.V the experimental description of Barron and Matthews is quite adequate. It remains then to clarify by experiment the origins and general properties of D.R.IV.

General Properties of D.R.IV

No *a priori* judgment may be made concerning the intramedullary activity responsible for the appearance, in a neighboring dorsal root, of the D.R.IV deflection. There exist three possible origins of that activity: (*a*) primary afferent fibers, (*b*) secondary neurons, and (*c*) both primary afferent fibers and secondary neurons. An advantageous means for study of D.R.IV is to compare its behavior in varied circumstances of stimulation, on the one hand with that of the D.R.I, II, III complex of primary afferent fiber origin, and on the other hand with that of D.R.V relating to secondary activity.

Antidromic Stimulation of Ventral Roots.—It is well known that a dorsal root potential of the type resembling D.R.V can be recorded, in the frog preparation, following antidromic stimulation of the motoneurons (1, 16). In Fig. 3 may be found records of dorsal root potentials evoked by stimulation with single shocks of an ipsilateral dorsal root (A and C), and of a ventral root (B and D), ipsilateral to, and of the same segment as, the dorsal root used for recording. Amplification was adjusted so that D.R.V and the antidromically evoked

potential that mimics it, would be of comparable recorded magnitude. At the left of Fig. 3 it can be seen that the two potential changes are reasonably comparable in general outline. In the early course of these potential changes, however, there are distinct differences seen to better advantage in C and D of Fig. 3, recorded with faster sweep speed. Present in record C, as the result of dorsal root stimulation is the characteristic sequence of deflections, D.R.I to V. By contrast the dorsal root potential consequent upon antidromic ventral root stimulation reveals between stimulus artifact and the onset of the prolonged negative deflection, some 12 msec. later, no change in the electrical state of the dorsal root. Thus the appearance in a dorsal root of a deflection re-

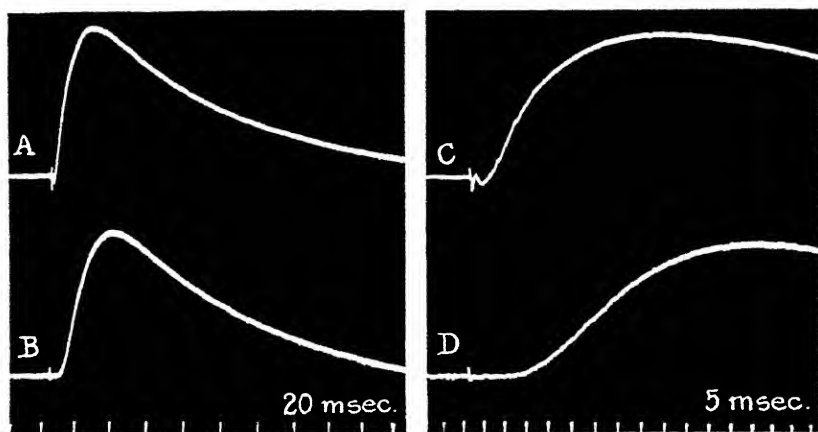


FIG. 3. Dorsal root potentials recorded, at two sweep speeds, following orthodromic stimulation of an adjacent dorsal root (A and C) and following antidromic stimulation of a ventral root (B and D). Bullfrog preparation.

sembling D.R.IV is not a necessary prelude to the appearance of a deflection resembling D.R.V. The observation demonstrates that activity in motoneuron somata, admittedly of a kind that results in a D.R.V-like wave, does not also polarize primary afferent fibers in the D.R.IV tempo, and suggests therefore that collaterals rather than somata may be responsible for D.R.IV.

Stimulation of Ipsilateral and Contralateral Dorsal Roots.—It is known from the observation of Barron and Matthews that the latency of D.R.V is longer when dorsal root potentials are recorded from a root contralateral to that stimulated than when recorded from an ipsilateral root. Fig. 4 illustrates a repetition of the observation to examine the antecedent potential deflections following ipsilateral (A) and contralateral (B) dorsal root stimulation. In each instance D.R.I, II, and III are present; so too is D.R.IV. As expected

the latency for D.R.V is widely different in the two recordings.¹ Despite this fact, virtually an identical temporal sequence exists between the D.R.I, II, III complex and D.R.IV, indicating the close association between these potential changes in circumstances that reveal a variable relation between D.R.IV and D.R.V.

Influence of Volley Size.—Fig. 5 presents a series of recorded dorsal root potentials arranged in order of increasing strength of stimulation. Record A of Fig. 5 was obtained by the use of a stimulus just over threshold strength; that

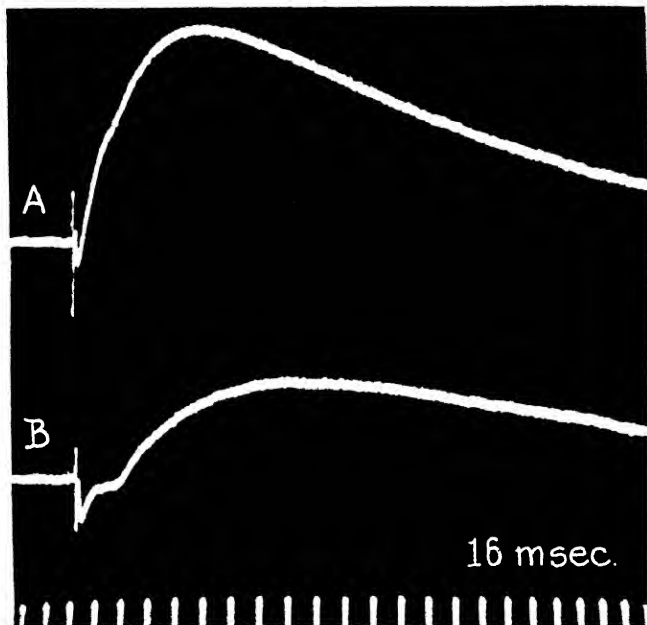


FIG. 4. Dorsal root potentials evoked by stimulation of an adjacent dorsal root (A) and of a contralateral dorsal root (B). Bullfrog preparation.

for record J was maximal. Dorsal root potentials evoked by contralateral dorsal root volleys were employed to avoid significant incursion of D.R.V upon D.R.IV with consequent distortion of the latter. Record A of Fig. 5 demonstrates that D.R.IV appears with D.R.I, II, III as threshold for dorsal root

¹ It is not possible to define with precision the latency of D.R.V. In ipsilateral recording, as may be seen in subsequent experiments, D.R.V would appear to begin as early as the peak of D.R.IV. Indeed, by interrupting the course of D.R.IV, D.R.V may determine in part the location of its peak. As an aside, recordings such as Fig. 4 A and Fig. 1 B, showing obvious discontinuities on the rising phase of D.R.V suggest that the potential change is not as simple as sometimes supposed (*cf.* also reference 11).

stimulation is exceeded, and records B to J show that the first four deflections of dorsal root potential increase in parallel with increase in volley size. In contrast to this parallel behavior D.R.V appears only after the antecedent deflections are well developed.

Experiment has shown that the clear-cut separation between the first four deflections of the dorsal root potential and the fifth that is obtained by varying the strength of contralateral dorsal root volleys is not so easily reproduced when ipsilateral dorsal root volleys are substituted for contralateral volleys. The fact is not surprising in view of the ever-present difficulties involved in segregat-

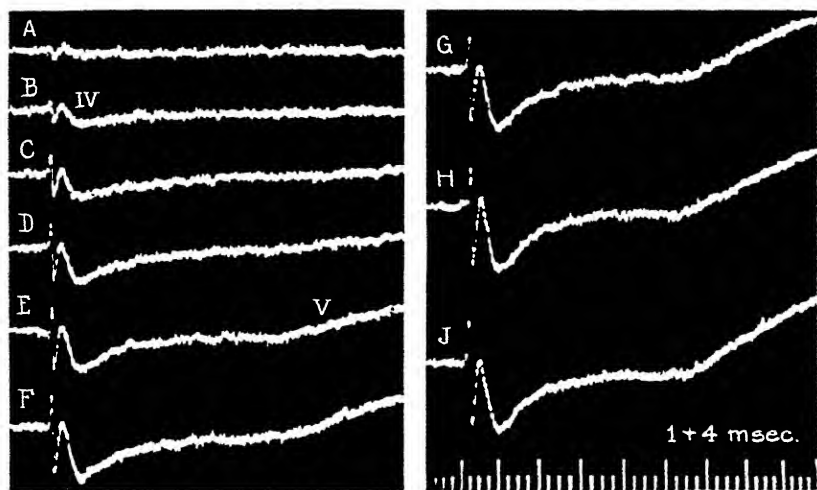


FIG. 5. Dorsal root potentials recorded, as in Fig. 4 B, from a root contralateral to that stimulated. Between each observation, A to J, stimulus strength was increased, that employed for A being just above dorsal root threshold, that for J being maximal. Sweep speed was such that only the onset of D.R.V appears in the recording. Bullfrog preparation.

ing one from another the various ipsilateral responses evoked by dorsal root stimulation (*cf.* reference 20, page 421).

When seen virtually free from interference, as in A, B, and C of Fig. 5, D.R.IV, in the bullfrog preparation, appears as a simple positive wave characterized by a rising phase of about 2 msec. duration and an approximately exponential decay to half-value in about 5 msec. It would seem a reasonable assumption that D.R.IV recorded from an ipsilateral root would exhibit a similar time course if, on recording, it were possible to divorce it from all interference from D.R.V. A number of experimental means have been tried, the most satisfactory being by the imposition of an asphyxial block. This is a simple procedure when studying the decapitate cat preparation, less so when employ-

ing the bullfrog preparation, due largely to the easy reversibility of the process in the former preparation.

Effect of Asphyxia on Dorsal Root Potentials.—In the experiment illustrated by Fig. 6, records of dorsal root potentials in a neighboring root were taken at regular intervals during a bout of asphyxia sufficiently prolonged to remove all trace of D.R.III, IV, and V. Representative records from the experiment have

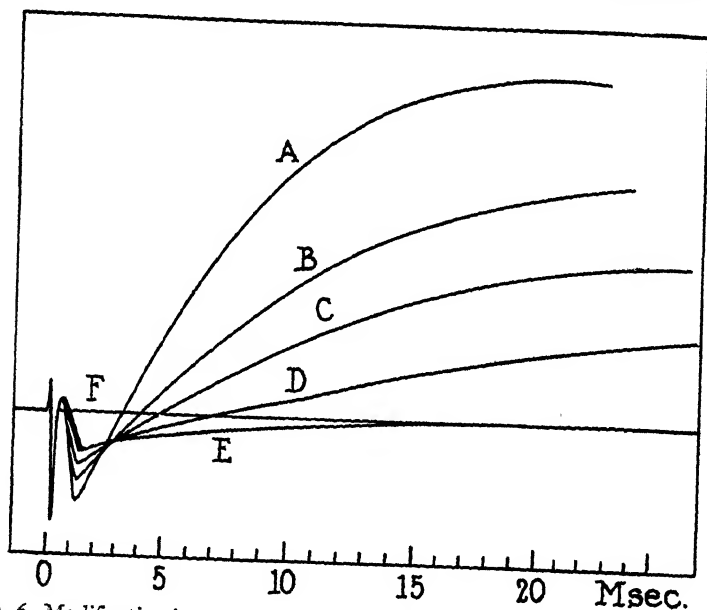


FIG. 6. Modification by asphyxia of dorsal root potentials recorded on a root adjacent to that stimulated. Cat preparation. A, normal dorsal root potential. E stage at which production of D.R.V is blocked (see also Fig. 7 B). F, stage at which only D.R.I and II appear. In the original records A to C, a small and progressively decreasing dorsal root reflex appeared; it has been omitted in reproduction. Details of the asphyxial block of dorsal root reflexes may be seen in Fig. 7.

been superimposed by tracing to illustrate successive stages of the asphyxial change, A being the normal dorsal root potential, E being the stage at which D.R. I to IV are still present but no trace of D.R.V remains, and F being the "final" stage in which only D.R.I and II are recorded. Thus of the five deflections D.R.I and II are the most resistant to asphyxia, D.R.III and IV are less so, and D.R.V is the most labile.

Fig. 7 contains actual records from another preparation of the normal dorsal root potential (A), and of the dorsal root potential at the stage of asphyxia in which D.R.V is removed. It is interesting in passing to note that the dorsal

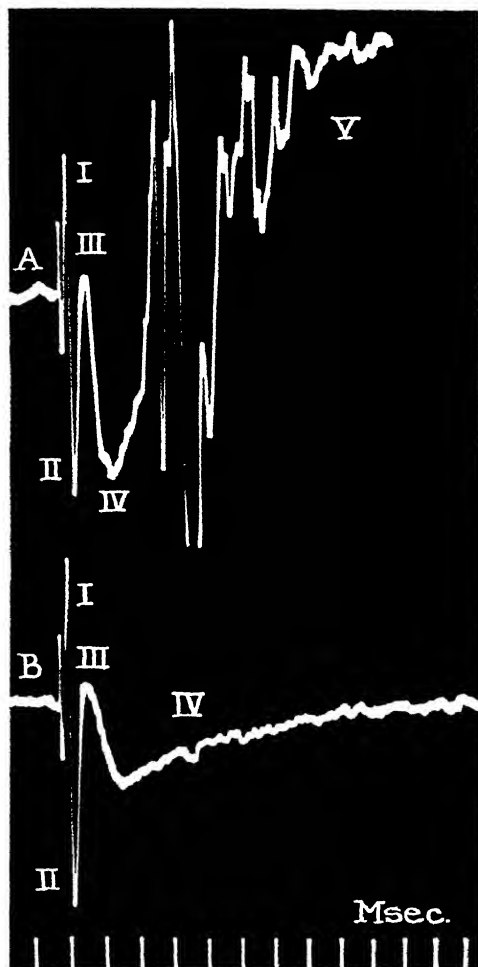


FIG. 7. Dorsal root potential recorded from a root adjacent to that stimulated. Cat preparation. A, normal, showing in this instance a large dorsal root reflex recorded in approximate diphasicity. The successive deflections of the dorsal root potential are identified by Roman numerals. B, to illustrate dorsal root potential as modified by asphyxia, the stage being comparable to that of Fig. 6 E. Deflections D.R.I, II, and III are not demonstrably altered from the normal. D.R.V and the dorsal root reflex have not been produced. D.R.IV is reduced and possibly slowed but on this latter point an unequivocal statement cannot be made. In this preparation, and in the stage of asphyxia represented in record B, it was seen that D.R.IV deflections evoked by simultaneous combination of two dorsal root volleys summed exactly. In accordance with the present analysis record B illustrates that part of the dorsal root potential referable to the activity of primary afferent fibers.

root reflex discharge, recorded diphasically on the rising phase of D.R.V in the normal condition, disappears along with D.R.V.

Seen cleared from D.R.V during the course of asphyxia, D.R.IV, in the cat, appears as a simple positive wave characterized by a rising phase of from 0.8 to 1.0 msec. in duration, and an approximately exponential decay to half-value in 3 msec., 2.6 to 3.5 being the observed variation in the series of experiments under consideration.

Summation and Occlusion of D.R.IV and D.R.V.—When two volleys of impulses enter the spinal cord through separate dorsal roots or rootlets, the dorsal root potentials being recorded in a third, and when the recorded dorsal root potentials evoked by the two volleys severally and together are compared, it is found that D.R.IV and D.R.V behave in a widely different manner. Results obtained from the bullfrog preparation and the cat preparation are qualitatively comparable.

If the two dorsal root volleys reach the spinal cord together over roots of opposite sides, the recorded D.R.IV deflection is equal, or very nearly equal, to the sum of the D.R.IV deflections resulting from the two volleys in isolation, whereas D.R.V suffers almost total occlusion. In Fig. 8 this finding is illustrated from an experiment with the bullfrog preparation. Represented by the broken lines (I) and (C) are the dorsal root potentials recorded, above with a fast sweep, below with a slow sweep of the oscillograph spot, following ipsilateral and contralateral dorsal root stimulation. The solid line (I + C) represents the dorsal root potential following combined stimulation.

If the two dorsal root volleys reach the cord both by roots ipsilateral to the root employed for recording, D.R.IV exhibits a degree of occlusion, greater in the cat preparation than in the bullfrog preparation, but at no time comparable to the subsequent occlusion of D.R.V. Fig. 9, constructed in the same fashion as Fig. 8, illustrates an experimental result in a bullfrog preparation. In the lower part of Fig. 9, it will be seen that the D.R.V deflection following simultaneous combination of two ipsilateral dorsal root volleys is little more than the greater of the two D.R.V deflections caused by the volleys in isolation, whereas there is only a slight failure of summation on the part of the D.R.IV deflections (upper part of Fig. 9).

In the normal cat preparation, when dorsal rootlets of the same side are stimulated in simultaneous combination and the dorsal root potential resulting is recorded from a third rootlet on the same side it is usual to find a degree of occlusion in D.R.IV potential. The deficit, as seen in the upper part of Fig. 10, is typically greater in the cat preparation than in the bullfrog preparation. If now the cat preparation be subjected to asphyxia until the stage is reached wherein D.R.V is abolished, but D.R.IV is still present, occlusion of D.R.IV disappears to be replaced by exact summation. This change in behavior is illustrated by Fig. 10, in which summation of D.R.IV in the normal state (above) and in the partially asphyxiated state (below) may be compared.

Conclusion as to the Origin of D.R.IV.—It seems quite clear that D.R.IV may have origin in more than one type of intramedullary activity. The fraction

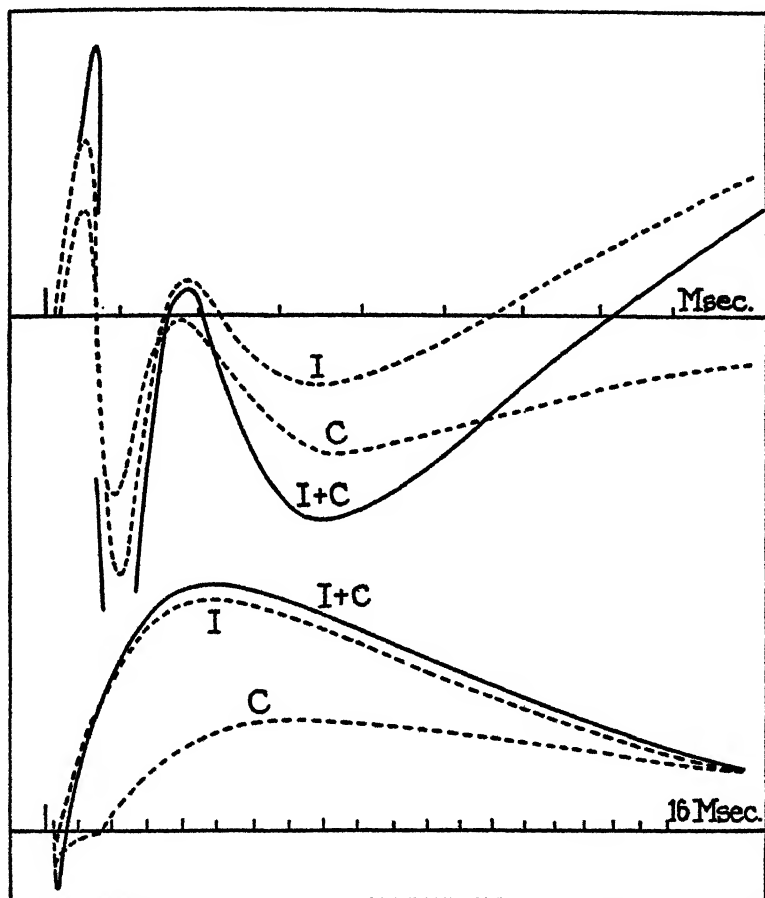


FIG. 8. Above, early course of dorsal root potentials elicited by stimulation of an ipsilateral adjacent dorsal root (I); by stimulation of a contralateral dorsal root (C); and by concurrent stimulation of both ipsilateral and contralateral roots (I + C) to illustrate exact summation of D.R.I, II, III, and IV. Below, full course of dorsal root potentials similarly evoked and similarly identified to illustrate almost complete occlusion of D.R.IV. Bullfrog preparation.

that appears in contralateral recording, or in ipsilateral recording when reactivity of the spinal cord is reduced by asphyxia is closely tied with events in primary afferent fibers. This non-occluding fraction of D.R.IV is most reasonably interpreted as being due to the activity of primary afferent fibers. However the evidence so far presented is compatible with the view that secondary

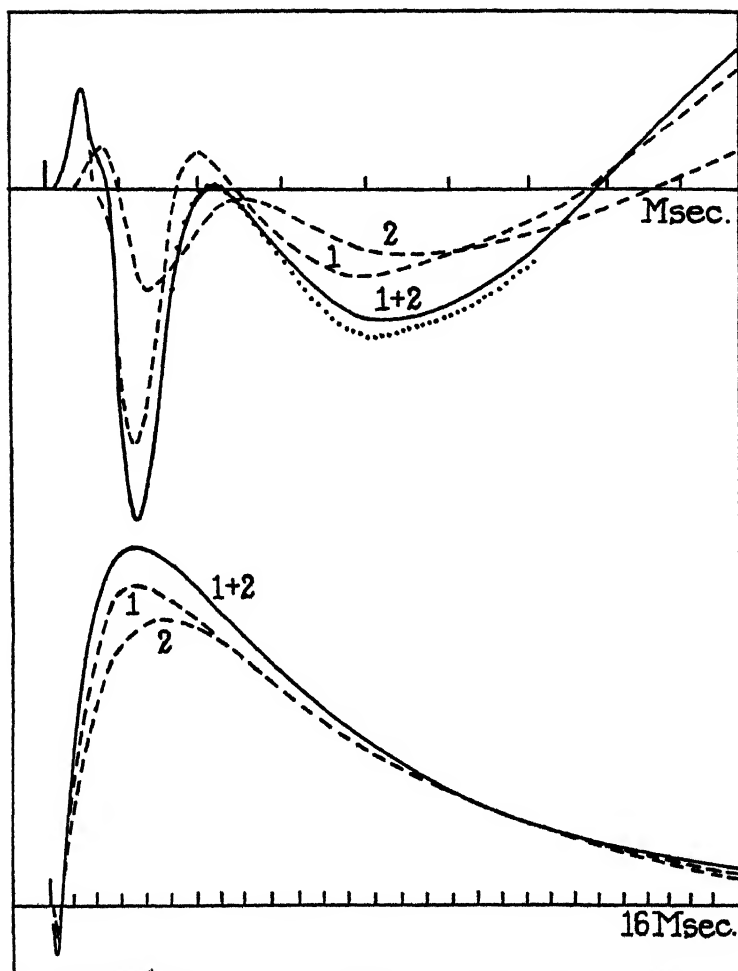


FIG. 9. Above, identified by broken lines, 1 and 2, is seen the early course of dorsal root potentials elicited by stimulation in isolation of two dorsal roots, the recording being from a third, all three roots pertaining to the same side. Identified by the solid line (1 + 2) is the dorsal root potential, similarly recorded, but evoked by concurrent stimulation. The dotted line, constructed by addition of 1 and 2 illustrates the slight occlusion of D.R.IV attending concurrent stimulation. Below, later course of the dorsal root potentials illustrating almost complete occlusion of D.R.V. Bullfrog preparation.

neurons are responsible for the non-occluding fraction of D.R.IV if one makes certain ancillary assumptions: that the responsible group of secondary neurons

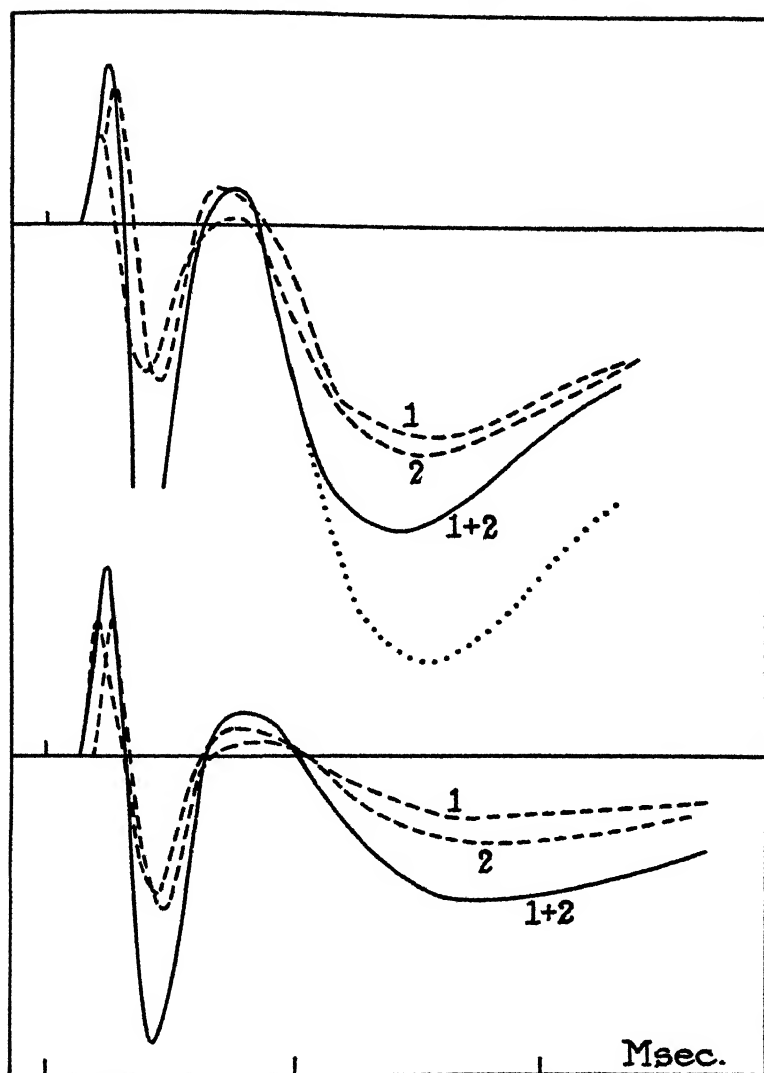


FIG. 10. Experiment similar to that illustrated in Fig. 9, but from a cat preparation. The broken, solid, and dotted lines have the same significance as that given in the legend of Fig. 9. Above, normal preparation, from which it is seen that D.R.IV is occluded to an extent typically greater than is found in experiment with the bullfrog preparation (but not to the same extent as is D.R.V in either preparation). It is significant that occlusion does not appear in the early course of D.R.IV either here or in Fig. 9. Below, as above, but in the partially asphyxiated state, sufficient for the removal of D.R.V. Note absence of occlusion.

would stand in "one-to-one" synaptic relation with primary afferent fibers, that the primary afferent fibers supplying those secondary afferent fibers do so without functional overlap, and that the activity of these secondary neurons does not lead to subsequent production of D.R.V potential. These assumptions being rather unlikely, it is concluded that the non-occluding fraction of D.R.IV results from polarization of primary afferent fibers by the activity of other primary afferent fibers. In the analysis of dorsal root potentials that follows certain consequences of the foregoing conclusion emerge. They may be put to test.

The remaining fraction of the D.R.IV potential, by the very fact that it exhibits occlusion, must be adjudged the result of activity in secondary neurons.²

Preliminary Considerations for an Analysis of Dorsal Root Potentials

An analysis of dorsal root potentials in a neighboring root must take into account a sequence of five deflections. The first three of these are *a priori* the result of activity in primary afferent fibers. The fourth apparently is due in part to the activity of primary afferent fibers, and in certain circumstances in part due to the action of secondary neurons. The last potential change of the sequence is, in its measurable extent, surely the activity of secondary neurons. It is profitable at this point to investigate dorsal root potentials in the light of what little is known of the neuronal architecture of the spinal cord and of recent advances in the general understanding of interaction between active and inactive fibers in nervous tissue (19, 24).

Considerations of an Anatomical Nature.—Primary afferent fibers on reaching the root-cord junction penetrate the dorsal columns with an approximately dorsoventral orientation and bifurcate in Y- or T-shaped fashion into longitudinally orientated fibers that ascend and descend the dorsal column. From the parent fibers and from their longitudinal projections, collaterals are directed into the gray substance. Of these collaterals some, derived from the parent fibers of greatest caliber, penetrate to the ventral horn. Others, in dense bundles, flow into the intermediate region. Still others curve on themselves to enter the dorsal horn by a ventral approach. In short the collaterals course through the gray substance in a variety of directions. Since each dorsal rootlet of a group contains a representative population of primary afferent fibers, it follows that the intramedullary projections of neighboring rootlets will lie in parallel array, not only in their longitudinal course within the dorsal column,

² The ingenious hypothesis of Barron and Matthews advanced to account for occlusion of D.R.V is relevant only to their assumption that D.R.V represents activity of primary afferent fibers. Once the assumption is seriously questioned, the older view that occlusion is a sign of response to convergent pathways (27) stands until proved incorrect.

but also in the gray substance, regardless of the direction taken therein by the individual collaterals. Now if of two neighboring rootlets one be stimulated and the other not, it further follows that, whatever their course in the cord, active and inactive fibers will lie in parallel. This is a fact of fundamental importance because it makes possible a treatment of dorsal root potentials in terms of relatively simple models of interaction between nerve fibers (24) without the necessity for intimate knowledge, which we do not now possess, of the exact spatial relationships between the many dendritic and axonal structures thrown into activity as a sequel to dorsal root stimulation.

Another important consequence of the parallel arrangement of primary afferent collaterals from neighboring roots concerns the polarization of them by the action of secondary neurons. Stimulation of a dorsal root, as is well known, leads to secondary activity as well as primary activity. Apart from the immediate consequences of impulse conduction by that dorsal root and its intramedullary projections, it follows from the fact of parallel orientation with intramedullary projections of a neighboring root that, whatever the orientation of the secondary neurons with respect to the primary projections, the net polarization of the latter by secondary activity, and so the resulting electrotonus in the two dorsal roots, will have similar duration and electrical sign, and approximately the same intensity.

A special case arises in consideration of dorsal root potentials led from contralateral roots. Primary afferent fibers from the two sides lie in parallel within the dorsal columns, but the collaterals diverge, each group to enter the gray substance of its side. The consequences of this fact emerge in subsequent discussion of ipsilateral and contralateral dorsal root potentials.

Considerations Relating to the Interaction of Neighboring Fibers in a Volume Conductor.—Since primary afferent fibers of a given rootlet may be considered as having similar properties, since they are activated synchronously by a single stimulus, and since the direction of their individual collaterals in the first approximation is inconsequential for the purpose of analyzing dorsal root potentials in a neighboring ipsilateral rootlet, they may be represented by a single fiber (A) of the shape illustrated by diagram A of Fig. 11. Likewise the primary afferent fibers of a neighboring rootlet may be represented by a similar fiber (N). Arrows in fiber A indicate, in diagram A of Fig. 11, the direction of impulse conduction from the extramedullary segment into the volume conductor (stippled) constituted by the spinal cord, thence in three directions, into the longitudinal fibers of the dorsal column and into the collaterals.

It is obvious that the electrical sign of electrotonic potentials in the extramedullary (E) segment of fiber N will depend finally upon the direction of current flow through the membrane of the intramedullary continuation of the parent fiber or I segment, and that this intramedullary continuation of the parent fiber lies in parallel with part of the intramedullary projection of fiber

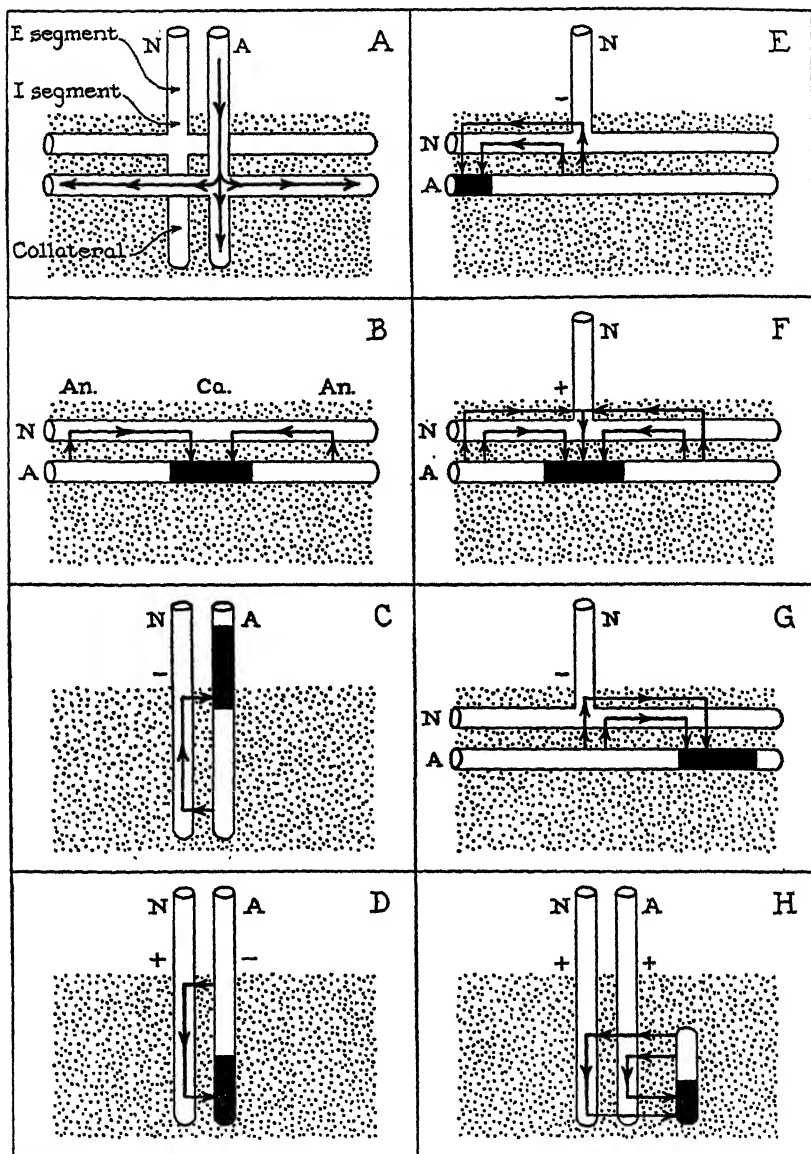


FIG. 11. Fundamental propositions relating to the analysis of dorsal root potentials. Fully described in text. In this figure and in Figs. 12, 14, and 17, fibers A and N represent active and neighboring fibers respectively; the shaded area represents the volume conductor; the black area of fiber A, in each instance, represents the impulse proper; while arrows indicate the external field about the active fibers. Plus and minus signs indicate the direction of electrotonus in the extramedullary (E) segment resulting from physical spread of current polarizing the intramedullary (I) segment.

A and at right angles to the remainder. It is convenient whenever possible to treat as separate problems the interactions resulting from impulse conduction in the parallel and 90° components of fiber A, synthesizing the two only as required by special considerations.

Fig. 11 presents in diagrammatic form the propositions necessary for a qualitative analysis of electrotonus in dorsal roots. Diagram B, recognizable as the fundamental proposition relative to the interaction of parallel neighboring fibers (19, 24), describes the changes that take place in resting longitudinal fibers as a volley of impulses travels along the dorsal column (26). Fiber N forms part of the external conductor of fiber A. Arrows indicate the three phases of membrane current in fiber A: outwards, inwards, outwards. In fiber N membrane current flows in a direction opposite to that in fiber A: inwards, outwards, inwards. The interaction is only detectable by measurement of threshold changes associated in fiber N with the anodal-cathodal-anodal succession of current flow through its membrane. It is important, because the principle is generally applicable, to note that an electrode placed near fibers A and N (*i.e.* on the dorsal column), and pitted against an electrode elsewhere on the preparation records the changes of membrane current in the active fiber A, not those in the neighboring fiber N.

Considered in connection with diagrams C and D of Fig. 11 are the interactions possible as the result of impulse conduction in fiber A from parent fiber to collaterals. Until the crest of the action reaches the root-cord junction current flow in the I segment of fiber N will be outgoing and the electrotonic potential in the E segment will be negative (diagram C). After the crest of the action passes the root-cord junction and until the collateral completes its recovery, current in the I segment will be ingoing and the resulting electrotonic potential of the E segment will be positive (diagram D). The situation described by diagrams C and D of Fig. 11, is none other than a special form of the situation at a fork in nerve (24, 13) and the proposed interaction therefore may be regarded as established in principle.

When an impulse travels in the longitudinal segment of fiber A the pattern of interaction between fiber A and fiber N is more complex, as may be appreciated by reference to diagrams E, F, and G of Fig. 11. To simplify the argument it is assumed for the present that the dorsal roots A and N are sufficiently far apart that interaction between the parent fibers is negligible. Further simplification by omitting the collateral of fiber N is possible since current flow there as the result of conduction in the longitudinal segment of fiber A is not of consequence to the I segment of fiber N. Illustrated by diagrams E, F, and G of Fig. 11 are three stages of interaction as the impulse in the longitudinal segment of fiber A approaches, resides at, and regresses from the level of dorsal root N. Current flow in the longitudinal segment of fiber N of course has the character defined by diagram B of Fig. 11, but the fact that the parent fiber of N enters the conducting medium has important additional consequences.

Briefly stated, membrane current will flow in the I segment of fiber N in a direction identical with that in the longitudinal segment A. Thus while the longitudinal segment of fiber N is polarized anodally by the outgoing current of A, the I segment is polarized cathodally, and the electrotonic potential of the E segment is negative (diagram E). Next in order, as the sink of current flow in the longitudinal segment of fiber A reaches the level of root N, the longitudinal segment of fiber N is cathodally polarized by the inward flowing current about A while the I segment is anodally polarized resulting in anelectrotonus of the E segment (diagram F). Finally, as the impulse in A recedes (diagram G) the entire sequence reverses again causing a catelectrotonic potential to appear in the E segment of fiber N.

In contrast to diagrams B, C, and D of Fig. 11, which are based on theoretical propositions that have already received experimental verification, the predictions of diagrams E, F, and G, as they apply to the I segment, lack prior experimental verification in a system which, by its evident simplicity, permits unequivocal demonstration of their validity. The following section is devoted to experimental confirmation of these latter predictions.

Diagram H of Fig. 11 serves to illustrate the only generally acceptable proposition concerning polarization of primary afferent fibers by the action of secondary neurons. Deliberately to discourage overly facile identification, in diagram H of Fig. 11, of the external polarizing source with any supposed or real structure, it is represented as a simple dipole devoid of anatomical meaning. Diagram H is but one of an infinite number of diagrams that might be drawn to illustrate the fact that, as long as fiber A and fiber N lie in parallel array, the result of polarization by a secondary source must be qualitatively similar in the two fibers.

On the Interaction, in a Volume Conductor, between Parts of Nerve Fibers Lying at Right Angles to One Another.—The theoretical argument advanced in connection with diagrams E, F, and G of Fig. 11 is susceptible of experimental verification in a nerve model. Fig. 12 illustrates the experimental arrangement and the expectation that electrotonus in the insulated segment will pass through three successive stages: cathodal, anodal, and cathodal respectively. It will be noted that the nerve model in many details differs from the spinal cord. For instance the absence of secondary neurons and collaterals removes any possibility that structures other than the longitudinal tract of fiber A could contribute to, or subtract from, the result. Furthermore, in the nerve model fiber N is L-shaped rather than T-shaped, giving rise at the angle to interesting differences in the course of interactions as may be appreciated by comparing Fig. 12, B, C, and D with Fig. 11, E, F, and G, but the qualitative aspects of polarization in the I segment of the primary afferent fibers and in its analogue in the nerve experiment should be similar.

Fig. 13 illustrates the result of an experiment, utilizing a bullfrog sciatic

nerve preparation in the manner defined in diagram A of Fig. 12. An electrode (A) was placed in contact with the "active" nerve near the fork, and at some distance in the volume conductor another electrode (B) was located. Since electrode A is situated at a distance from the point of entry of impulses into the volume conductor, electrodes A-B should record, as a triphasic deflection, the

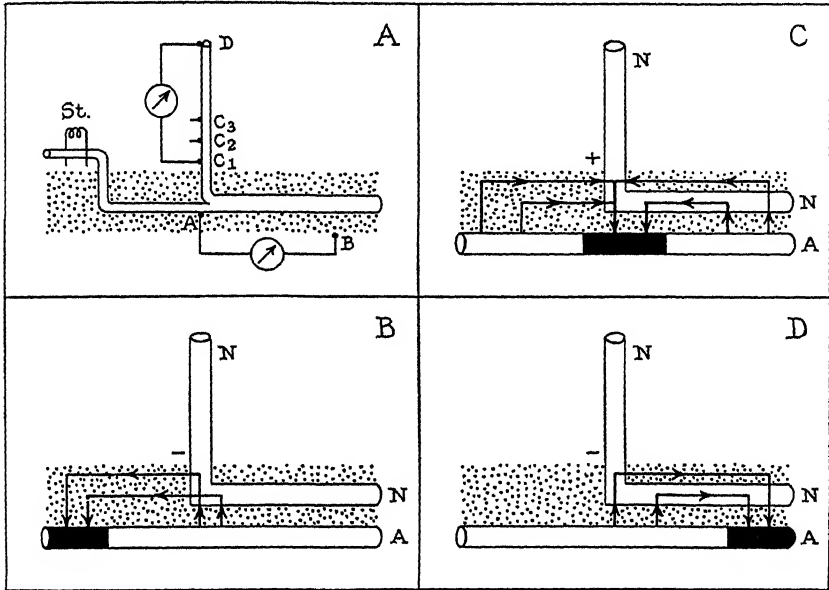


FIG. 12. In diagram A is illustrated the experimental arrangement for demonstration in nerve of interaction between fibers orientated at 90° to each other. Bullfrog sciatic nerve with its two principal subdivisions. St, location of stimulating electrodes. A-B, leading arrangement to record impulses in the active fibers. C-D, leading arrangement to record electrotonus in the inactive fibers, positions C₁, C₂, C₃ of electrode C serving to demonstrate the electrotonic decrement of potential changes in the inactive fibers. Diagrams, B, C, D, to illustrate the anticipated course of interaction as impulses in active fiber A approach, reside at, and depart from the region of 90° orientation.

passage of impulses evoked by stimulation through electrodes St. The "neighboring" branch of the sciatic nerve was drawn up into oil, in such a manner as to leave a short stretch near the fork within the volume conductor to form with the active branch a 90° angle. Electrodes C and D were placed on the insulated segment, the former held by a micromanipulator so that it could assume the successive positions C₁, C₂, C₃. . . , in order to record electronic propagation into the insulated segment and demonstrate its decremental character.

Record A of Fig. 13, obtained by use of the leading electrodes A-B (Fig. 12),

contains the familiar triphasic deflection expressing changes in membrane current during the passage of a volley of impulses (23). The sequence, as antici-

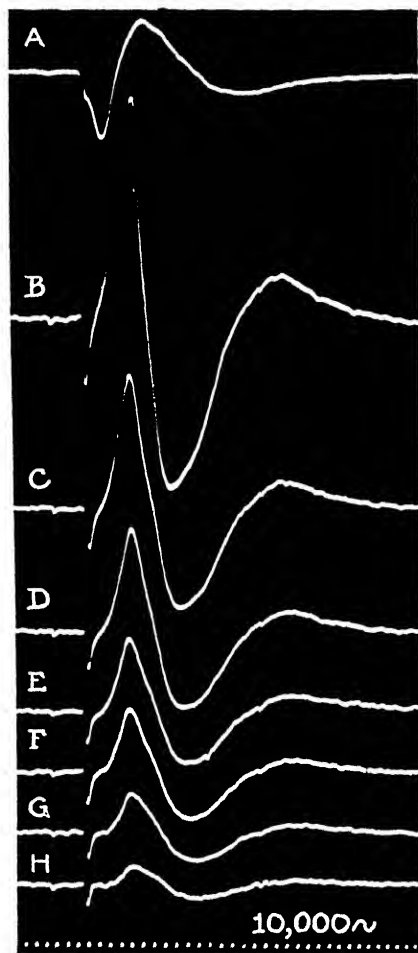


FIG. 13. Experimental findings relating to the recording arrangement of Fig. 12 A and in confirmation of the interactions proposed in Fig. 12, B, C, and D. Record A, obtained by means of electrodes A-B in Fig. 12 A. Records B to H, obtained by means of electrodes C-D in Fig. 12 A, the successive records being made with electrode C at locations C_1 , C_2 , C_3 ,

pated is positive-negative-positive. Illustrated in B to H of Fig. 13 are records obtained by the use of electrodes C-D, the successive observations recording the change as electrode C is moved, in millimeter steps from C_1 to C_2 to C_3 For observations B to H of Fig. 13 negativity at electrode C relative to elec-

trode D is recorded upwards. It is clear that the electrotonic currents in the insulated segment exhibit in succession an outward, an inward, and an outward direction. In accordance with expectation, test stimulation, in the neighborhood of C_1 , of the insulated segment reveals there a succession of enhancement, depression, and enhancement.

One need hardly emphasize the purely qualitative nature of the present analysis of interaction between fibers, undertaken for the sole purpose of verifying the physical possibility of the previously unsubstantiated propositions (Fig. 11, E, F, and G) necessary for analysis of dorsal root potentials.

Analysis and Interpretation of Dorsal Root Potentials

Analysis of D.R.I, II, III.—Since the first three deflections of the dorsal root potential bear a remarkable resemblance to the intramedullary spike potential that signals conduction of a volley within the dorsal columns, there can be little doubt that interaction of the type outlined in diagrams E, F, and G of Fig. 11, by accounting for the electrical sign of these deflections, contains the elements of a satisfactory interpretation of their origin. However, in order to simplify the initial presentation, in Fig. 11, E, F, and G, of the concept of interaction between fibers lying at right angles to one another, a limiting case was chosen, that in which the dorsoventral components of fibers A and N are too far apart for effective interaction. Fully to describe the origin of D.R.I, II, and III it is necessary to present the other limiting case, that in which the dorsoventral components of fibers A and N are so close together that the root-cord junction of fiber N is in effect at the point of entry into the volume conductor of impulses in fiber A. This limiting case, presented diagrammatically in Fig. 14, is particularly interesting, for, despite the fact that the intramedullary spike potential recorded at the root-cord junction of fiber N (and naturally of fiber A) is diphasic, negative-positive (*cf.* reference 23), D.R.I, II, and III are all present in the E segment of fiber N.

For construction of Fig. 14 it will be seen that no propositions have been employed other than those advanced in Fig. 11. When fibers A and N are immediately adjacent (diagram 14 A) it is the entering impulse in the parent fiber A that determines outward flow of current in the I segment, and the appearance of catelectrotonus in the E segment of fiber N. After the crest of the action enters the cord and until the beginning of recovery in the longitudinal segment of fiber A (diagram 14 B) three sources of current flow, S_1 , S_2 , and S_3 , exist in fiber A, all of which are capable of causing current to flow inward through the I segment of fiber N with resulting anelectrotonus in the E segment. Finally, as recovery of the longitudinal segment of fiber A progresses (diagram 14 C), current again will flow outwards in the I segment of fiber N producing catelectrotonus in the E segment.

It is obvious that, in most instances, the recorded D.R.I, II, and III deflections will represent current flows of complex origin intermediate between the

two limiting cases. No useful purpose would be served by carrying this analysis further, since an individual problem is raised by each experimental arrangement for the recording of dorsal root potentials.

Analysis of the Primary Fraction of D.R.IV.—Following a study of the properties of D.R.IV, it was concluded above that a considerable fraction of this deflection, the non-occluding or primary fraction, could most reasonably be attributed to the activity of primary afferent fibers. If this be true, it further

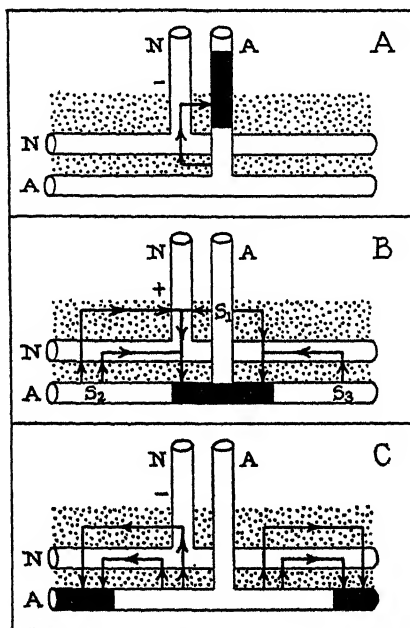


FIG. 14. Diagrams to illustrate one limiting case, the other being illustrated in Fig. 11, E, F, and G, for fiber interaction determining the production of the D.R.I, II, and III deflections of dorsal root potentials.

follows that the responsible activity must be centered in the collaterals, since it is unreasonable to attribute activity having the character of D.R.IV to simple conduction along tract fibers the known properties of which so closely resemble peripheral nerve.

Recourse to the fundamental propositions of Fig. 11, reveals that D.R.IV could only result from the current flows described in diagram D and diagram H. That fraction of D.R.IV that results from activity in primary afferent collaterals (the non-occluding or primary fraction) must arise in the manner of diagram 11 D, the occluding fraction must arise in the manner of diagram 11 H.

Considering the positive sign and prolonged duration of the primary fraction

of D.R.IV, it is possible to predict that this potential deflection represents a persistent negativity in the primary afferent collateral A, resembling rather the residual negativity at a nerve block described by Lorente de N6 (22) although in this instance the gradient must be relatively much more powerful. This prediction carries with it a consequence that may be put to experimental test. Since the parent fibers certainly have recovered during the period of D.R.IV, it would be expected that the supposed residual negativity of the active collaterals should be propagated backwards into the parent fibers and would appear in the active dorsal root as a negative deflection at the more proximal of two recording leads placed thereon.

Now it is known that the dorsal root potential of an active root is a complex event (*cf.* discussion in relation to Fig. 1 A). A negative potential difference of the sort postulated would be written upon the fiber potentials of the root itself and would be overlaid by the large negative potential difference paralleling D.R.V. The required procedure for its demonstration then rests upon the fact that D.R.IV during asphyxia is less labile than D.R.V, and more labile than the potentials relating to spike conduction (including in this instance the after-potentials of the active dorsal root fibers in contact with the recording leads). Polarization potentials, the result of the stimulating current, should not change by virtue of intramedullary changes during asphyxia. With these propositions in mind, one may consider the observations recorded in Fig. 15, taken from the same experiment as was Fig. 6, but showing changes wrought by asphyxia in the active rather than in a neighboring root.

The records reproduced in Fig. 15 were made during the course of recovery from a period of asphyxia. Figure 15 A illustrates the course of dorsal root potential that was maintained constant over a period of some minutes, and thus is taken to be the contribution from extramedullary sources. Following re-establishment of respiration (and circulation³) the dorsal root potential changed first to the form illustrated in Fig. 15 B, and later recovered through the stages represented by records C to G. Fig. 15 clearly indicates that, in addition to the extramedullary components of the dorsal root potential in an active root, there are two gradients of negativity, exhibiting differing temporal course and differing susceptibility to asphyxia. The more resistant of these, as judged by earlier recovery after asphyxia, is represented by the difference in potential level between records A and B of Fig. 15. When plotted on an isopotential base line (dotted line in Fig. 15) the potential difference represented by (B-A) is seen to match closely, but with opposite electrical sign, the D.R.IV deflection recorded in similar conditions from a neighboring root (Fig. 6E).

Identification of the early negative potential difference in an active root with

³ In the course of the experiment from which Figs. 6, 15, and 18 were prepared in each instance the asphyxia was so severe that cardiac arrest resulted. Circulation was restored by massaging the chest.

the positive D.R.IV of a neighboring root is greatly facilitated by means of simultaneous recordings from the two roots in question. Fig. 16 presents an experiment in which asphyxial changes in the dorsal root potential of the active root (A to D above) and of a neighboring root (A to D below) were recorded simultaneously with the aid of a twin-beam oscillograph. In each instance A, B, C, and D illustrate successive changes in the dorsal root potentials from the onset of asphyxia (A) until only D.R.I and D.R.II remained in the dorsal root potential of the neighboring root (D). Records A, B, and C illustrate the

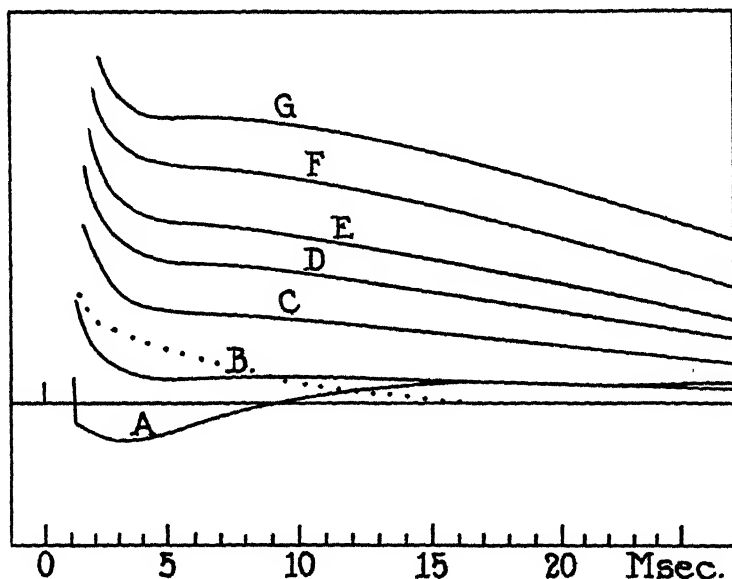


FIG. 15. Modification by asphyxia of dorsal root potentials recorded from a stimulated root. A, course of dorsal root potential after prolonged asphyxia. B, first stage of recovery. C to G, subsequent stages of recovery to normal. The dotted line plots on an isoelectric baseline the potential difference between records A and B

progressive loss of D.R.V and its homologue in the active root. The further change (D) in the active root, consisting of loss of the early negative potential, exactly parallels the loss of D.R.IV in the neighboring root. For this reason it is concluded that the two potential changes indeed are associated. This being so it is a necessary consequence of their electrical signs that the causal activity resides not in secondary neurons, but in the active primary afferent fibers.

Concerning Potentials in the Terminal Regions of Presynaptic Fibers.—The foregoing observations call for the existence, following the arrival of impulses at the primary afferent endings, of an enduring flow of current in the direction from parent fibers to terminal regions. In the absence of interfering secondary activ-

ity such a current flow would be recorded by a microelectrode appropriately situated in a nucleus of termination as a negative wave detectable for nearly 15 msec. and decaying over an approximately exponential course to half-value

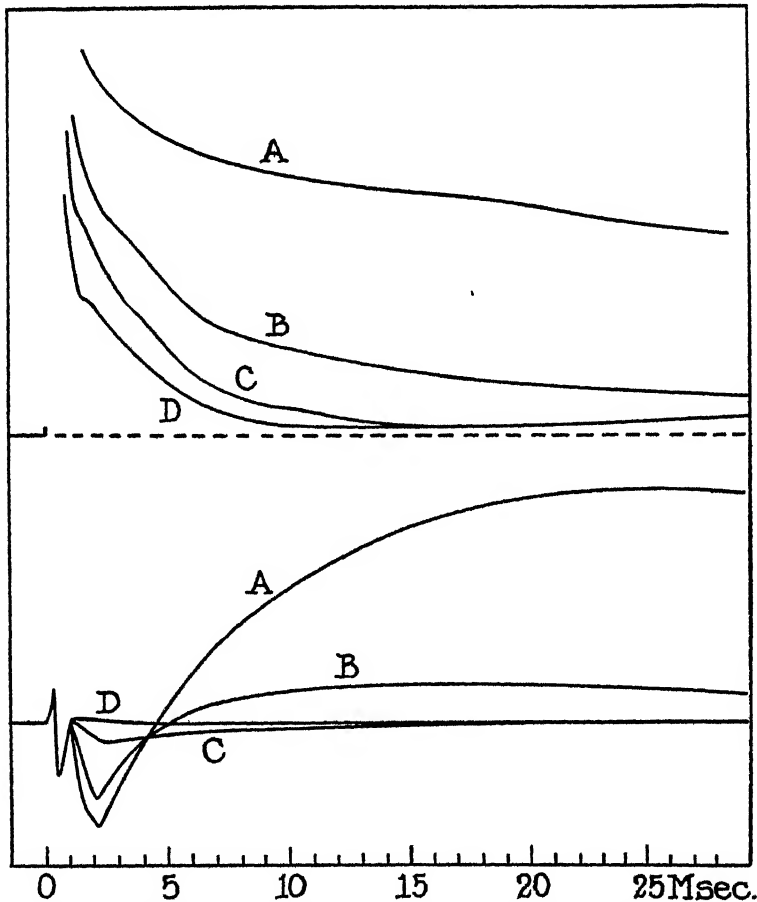


FIG. 16. Simultaneous recording of dorsal root potentials from the stimulated root (above) and a neighboring root (below) to show, in A, B, and C parallel progress in the two roots of asphyxial block of the D.R.V deflection and its homologue, but in particular to illustrate, by comparison of the potential level of C and D in each case, the parallel loss of early negativity in the active root and of D.R.IV in the neighboring root.

in slightly less than 3 msec. In experiments that fulfill the stated conditions, Brooks and Eccles (7) have recorded such a potential to which they have given the name "focal synaptic potential." It is their hypothesis that the sinks of

current flow giving rise to this recorded potential difference lie in the motoneuron somata, the sources at the motoneuron axons, whereas the present experiments demand that a similar if not identical potential difference must be recorded by virtue of sinks of current flow located in the presynaptic fibers at or close to the endings, the sources being located in the presynaptic fibers at some greater distance from the endings.

Potentials referable to activity in the terminal regions of presynaptic fibers have been recorded, but not free from interference by secondary activity, in the oculomotor nucleus by Lorente de Nó (22) and in the quadriceps nucleus of the spinal cord by Renshaw (25). While postulating that the " β deflection" of Renshaw is identical with their "focal synaptic potential" Brooks and Eccles (7) have disputed Renshaw's conclusion that his " β potential" is produced by the terminal portions of the presynaptic fibers. Considering the present evidence, however, it would seem that Renshaw's surmise undoubtedly was correct.

It should be emphasized that the recording by microelectrode within a motor nucleus of a potential change of the type under discussion does not yield of itself sufficient information by means of which to decide whether the recorded potential is referable to presynaptic or postsynaptic structures. Since the potential change in the nucleus is of negative sign it follows that the sinks of current flow are there. It likewise follows, in the monosynaptic system, that the sources must be either in the motoneurons, or in the primary afferent fibers. On the assumption that the motor axons supply current to the somata during the "synaptic potential," Brooks and Eccles (7) regard the existence of a ventral root "synaptic potential" as proof of the postsynaptic origin of the "focal synaptic potential." But the sources of current flow that determine the course of the ventral root synaptic potential outlast by two to three times the sinks that determine the "focal synaptic potential" (7). On the contrary the sources in primary afferent fibers that determine the appearance in an active dorsal root of the early negativity (Figs. 15 and 16) have a duration comparable to that of the sinks determining the "focal synaptic potential." In view of this last fact it seems unlikely that electrotonic slowing assumed to occur in the intramedullary course of the ventral root fibers (14) could account for the discrepancy between "focal synaptic potential" and ventral root "synaptic potential." In the circumstances the simplest conclusion must be that the two "synaptic potentials" are manifestations of different systems of current flow. One may assume a causal relationship while recognizing the obscurity of its nature.

It is important to recognize that the fraction of dorsal root potential of primary origin (as seen in Fig. 7 B) cannot reflect in precise detail all phases of the potential sequence in the terminal regions of the presynaptic fibers although in general they are not dissimilar except in electrical sign. To simplify the argument the reasons for this fact are presented diagrammatically in Fig. 17. From a study of diagrams A, B, and C of Fig. 17 it will be seen that from the time that

the crest of the action in fiber A reaches the volume conductor of the spinal cord (immediately after the condition represented by diagram A) until it reaches the presynaptic terminals (diagram C) the polarization of the I segment of fiber N, and consequently the dorsal root electrotonus, is caused by a flow of current different from that surrounding the collaterals (diagram B). Consequently it is only after the crest of the action reaches the presynaptic ter-

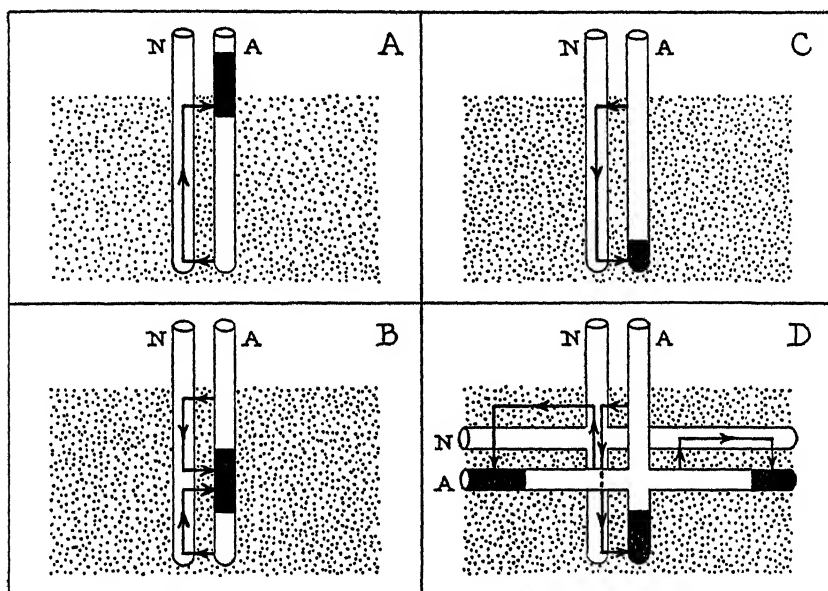


FIG. 17. Diagrams to illustrate the fact that D.R.IV and the potential changes of active primary afferent collaterals for part of their time course could be related to the same current flows, but that also for part of their time course they cannot be so related, the dorsal root electrotonus being determined by a current flow different from that surrounding the collaterals (diagram B). Diagram D illustrates the manner in which the I segment may be subjected to opposing current flows during the course of the D.R.III deflection.

minals (if, indeed, it does) that D.R.IV and an "ending potential" could be compared in detail.

Another factor that must contribute to differences of the sort under discussion may be mentioned in connection with diagram D of Fig. 17, for it provides in addition an understanding of the fact that D.R.III uniformly has not been of as great amplitude as one might reasonably have expected. Diagram 17 D shows how, once the primary impulse conducts beyond the parent fiber, and until recovery following the impulses that continue in the longitudinal segment of fiber A is complete, the I segment of fiber N is subjected simultaneously to

anodal and cathodal flows of current. In short D.R.III is written, not upon an isoelectric base line, but interrupts, so to speak, an otherwise more or less continuous anelectrotonus consisting of D.R.II and D.R.IV.

The Occluding or Secondary Fraction of D.R.IV.—Although there can be no reasonable doubt but that the occluding fraction of D.R.IV results from the activity of secondary neurons, there is no direct evidence bearing on the mode of its production. Indirect evidence, however, would seem to justify the view that the occluding fraction is produced by essentially the same type of activity on the part of the secondary neurons as that which in primary afferent fibers are responsible for the appearance of the non-occluding or primary fraction. The argument is as follows: Activity in primary afferent fibers, which is entirely axonal, produces a D.R.IV potential, but no D.R.V potential. Secondary activity, in which, among the internuncial pools, both axons and somata are equally involved, produces both a D.R.IV potential and a D.R.V potential. Secondary activity elicited by antidromic volleys, in which case the activity in the main must be referred to somata, produces no D.R.IV potential but (in the bullfrog) results in a dorsal root potential indistinguishable from D.R.V. Finally, given a parallel orientation of secondary axons and primary afferent fibers (as is found, for instance, between the axons of neurons of the intermediate nucleus and the "reflexomotor" collaterals) the essential anatomical requirement is satisfied, and activity of secondary collaterals becomes a sufficient explanation of the occluding fraction of D.R.IV.

It has not been possible in a satisfactory manner to distinguish among the components of the dorsal root potential in an active rootlet one that corresponds to the occluding fraction of D.R.IV in a neighboring rootlet.

Ipsilateral and Contralateral Dorsal Root Potentials.—Dorsal root potentials recorded in an adjacent ipsilateral root and in a contralateral root differ in two important respects: (a) The secondary fraction of D.R.IV is relatively large in ipsilateral recording, relatively small or absent in contralateral recording, and (b) a D.R.V deflection appears contralaterally only after considerable latency and at a time when ipsilateral D.R.V may have reached 60 to 70 per cent of peak amplitude. Both of these differences express the fact that secondary activity centered in the gray substance of one-half of the spinal cord does not in a significant degree polarize primary afferent fibers of the other side. There could be two reasons for the fact, both anatomical. It may be, at the distances involved, that the field generated by active secondary neurons is too weak to cause appreciable effect, or it may be that the orientation of the field is such that little or no net polarization of contralateral primary afferent fibers results. The two factors are not in any way mutually exclusive.

Since the primary afferent collaterals on the two sides of the cord diverge and course laterally to splay out into the gray substance, it is improbable that activity confined to the endings of one group could influence the other group any

more than could secondary neurons of the contralateral gray substance. The fact that a D.R.IV deflection is recorded contralaterally therefore can only be explained by the additional fact, demonstrated in Figs. 15 and 16, that the residual negativity of primary afferent collaterals propagates itself backward into the I segment (and hence presumably also into the longitudinal fibers) which parts of the primary neurons are suitably orientated in sufficient proximity (as shown by the appearance of D.R.I, II, and III in contralateral recording) to permit the necessary interaction to take place.

The appearance of a D.R.V deflection in contralateral recording apparently depends upon the spread, by decussation through commissural neurons, of activity from one to the other half of the spinal cord.

On the D.R.V Deflection.—It has already been stated that the present experiments add little to what is known of the D.R.V deflection. Since this prolonged negative wave appears in active and neighboring roots alike with the same electrical sign, it follows from the propositions considered in connection with Fig. 11 that secondary neurons are responsible for the polarization that produces this deflection. To this extent the present experiments are in agreement with the interpretations of Bonnet and Bremer (2, 3) and of Eccles and Malcolm (16). Furthermore, for reasons that have been discussed, it seems likely that the activity of somata rather than that of axons is the causal agent.

The observation at times has been made, initially by Barron and Matthews in their original description of "the dorsal root potential" (1), that the D.R.V deflection evoked by a dorsal root volley parallels in time course the positive intermediary potential (17) similarly evoked. It would seem that this is a significant fact, and, as a first approximation, it would further seem justified to assume as did Barron and Matthews, that the two phenomena are intimately related despite the obscurity that at present surrounds the relationship.

D.R.IV and Negative Cord Potential.—During the analysis of the D.R.I, II, III complex it was seen that these deflections bear a reasonably definable relation to the intramedullary spike potential of the cord potential. Likewise there exists a close similarity, but ill defined relationship between D.R.V and the positive intermediary potential. The similarity between D.R.IV and negative intermediary potential at first sight, however, is neither close nor well defined. Some observations on cord potentials during the course of asphyxia have interest in this connection. Illustrated in Fig. 18 are records, superimposed by tracing, of cord potentials recorded at various stages of asphyxia by means of electrodes placed one upon the dorsum of the cord, the other at a distance on non-nervous tissue. The records are from the same preparation as that from which Figs. 6 and 15 were obtained. Record A of Fig. 18 represents the normal cord potential, B to E, successive stages in the asphyxial effect. From these recordings it is quite clear that the negative intermediary potential is divisible into two fractions, one associated with and succeeded by positive intermediary

potential, the other not. The latter, seen in isolation in record E of Fig. 18, has properties similar to those of the primary fraction of D.R.IV and the early negativity in an active dorsal root, including virtually identical duration and regression: it would appear to share with them a common origin.

The problem raised by the electrical sign of the presumably primary fraction of the negative intermediary potential (Fig. 18 E) is different from that involved in the study of similar deflections in dorsal roots for the precise orientation of the individual collaterals within the volume conductor of the spinal cord

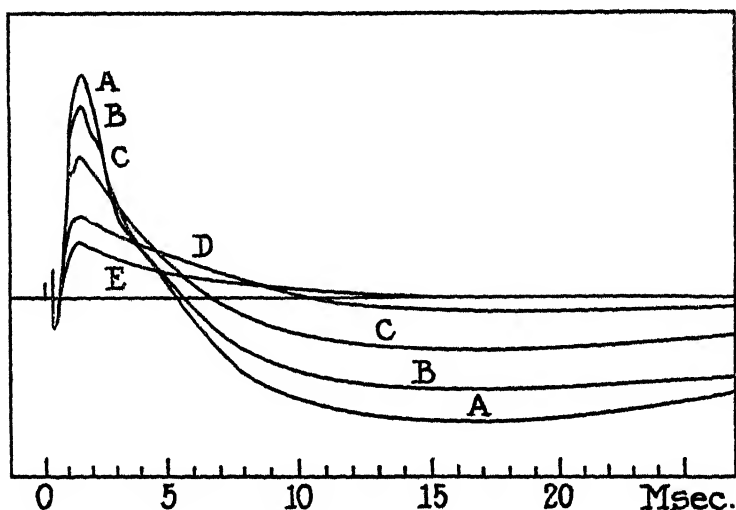


FIG. 18. Cord potentials recorded with one electrode on the cord dorsum, the other on non-neural tissue, to illustrate progressive stages, A to E, in the course of modification by asphyxia. The negative intermediary potential contains two components separable by asphyxia, one of which (E) in many ways is comparable with D.R.IV.

can no longer be neglected. In other words a unique solution is only possible following a study of the fields about active primary afferent fibers in the light of knowledge as to the precise orientation of the active fibers. The knowledge not being available the problem cannot as yet be solved.

The other fraction of negative intermediary potential is clearly the result of internuncial activity (17), possibly on the part of the interneurons responsible for the secondary fraction of D.R.IV. There is not much resemblance between these potentials, a fact that could mean among other things merely that the aspect of internuncial activity that dominates the intermediary potential does not possess the appropriate spatial orientation to result in a net polarization, one way or the other, of the I segment of primary afferent fibers. Since the action of secondary axons is the likely cause of the polarization producing the secondary fraction of D.R.IV, one might suppose that activity in inter-

nuncial somata predominates in the cord lead, and so accommodate the discrepancy.

On the Relative Susceptibility to Asphyxia of Various Parts of the Reflex Arc.—The present experiments utilizing asphyxia have provided an opportunity to study the relative sensitivity to asphyxia of the several structures encountered by impulses on their way through the spinal cord. To recapitulate the experimental observations, in each instance D.R.V and the occluding (secondary) fraction of D.R.IV disappear together, leaving for a short period the D.R.I, II, III deflections and non-occluding fraction of D.R.IV (Figs. 7 and 10). Next to fail with continuing asphyxia are the remainder of D.R.IV and D.R.III. A resistance to asphyxia comparable to that of peripheral nerve characterizes the D.R.I and D.R.II deflections. These observations can only mean that the ability of somata to generate impulses in response to presynaptic volleys is the first asphyxial failure, although to judge from the observations of Brooks and Eccles (8) the somata (of motoneurons) should still be able to respond to antidromic impulses. Subsequent and conjoint failure of D.R.III and the non-occluding fraction of D.R.IV indicates that the "weak point" of the primary afferent fibers is the region where parent fibers branch to form longitudinal fibers and collaterals, rather than the collaterals or terminals themselves.

An Analysis of Synaptic Excitatory Action

It has been shown (14, 20) that the facilitatory action of a near synchronous presynaptic volley in a monosynaptic reflex system can be detected for nearly 15 msec., the decay of facilitation being an approximately exponential regression to half-value in 2.8 msec. (20). At the time that the prolonged facilitation was documented reason was given (20) for supposing it to be the expression of a process additional rather than alternative to the brief excitatory action of earlier descriptions. Hence, and because of certain analogies with phenomena at a block in nerve (22), the prolonged facilitation was called *residual facilitation*. In other words it was concluded that presynaptic impulses exert upon postsynaptic neurons two actions: the initial (or detonator⁴) action and a

⁴ Attention must be brought to the fact that the term employed herein, and previously (20), is detonator *action*, not detonator *response*, since, to the extent that one term has been substituted for the other, the earlier paper (20) has been misquoted (15). By detonator action is implied, in accordance with Eccles' first use of the term (reference 12, page 6), a brief excitatory action exerted by presynaptic impulses. The term detonator response, again in accordance with original usage (reference 12, page 11), implies an action *sui generis* on the part of the postsynaptic neuron presumed to mediate between the excitatory action of presynaptic impulses and the generation of postsynaptic impulses. If, however, the term detonator action is to be construed in such a way (*cf.* reference 12, page 17) that it includes a detonator response, then present use (20, and above) has been in error.

residual action. Despite differences in detail, this conclusion in itself is nothing more than a reiteration of the views advanced by Bremer (4). From the foregoing experiments and consideration it is now concluded that the residual action of presynaptic impulses consists of a current flow about presynaptic collaterals during the period of residual negativity, and that such current flow about primary afferent collaterals is a sufficient agent to account for the phenomenon of residual facilitation in monosynaptic reflex pathways.

None of the experimental material upon which this paper is based bears on the nature of the event (initial excitatory action) by means of which the transmission of impulses is secured. As a matter of fact two important properties of the initial action, its brevity⁵ and sharp spatial decrement (22), preclude the possibility of electrotonic propagation into dorsal roots. However, there is still no reason to doubt the existence of an initial action to which the residual action is appended, and indeed the most recent experiments of Brooks and Eccles (10) have yielded facilitation curves that indicate a brief early phase preceding or superimposed upon the residual facilitation. Furthermore, the conditions that Brooks and Eccles have found necessary for demonstration of the initial facilitation are in full accord with the notion of sharp spatial decrement. Although Brooks and Eccles now accept the postulation of two phases of facilitation in monosynaptic reflex arcs, their interpretation as to origins is necessarily quite different from that here presented, being founded upon the fundamental assumption (7-10, 12-14) that current flow at the presynaptic endings has essentially the brief duration of axon spikes in peripheral myelinated fibers. As a consequence (and in consideration of "synaptic potentials") it was necessary further to assume that the more prolonged phenomenon, by whatever name it may be called, resides in the postsynaptic neurons. Now that the assumption concerning brevity of the presynaptic action is demonstrably untenable, the hypotheses of excitation (10, 13), inhibition (9), and of the origin of the dorsal root potential (16) predicated upon it appear unnecessarily complicated.

Although the major emphasis is now placed upon presynaptic events as agents for transmission and facilitation (not to speak of direct inhibition) it must not be supposed thereby that membrane changes do not occur also in postsynaptic neurons when acted upon by presynaptic impulses. However, in the presence of active presynaptic changes during orthodromic reflex activity, it seems highly unlikely that membrane changes of postsynaptic origin would be revealed by recording at the region of synapsis unless and until the postsynaptic neurons respond in turn with conducted impulses. In the absence of discharge, therefore, the demonstration of threshold change, as by the use of monosynaptic test

⁵ To avoid misunderstanding it is well to note that brevity is a factor only in the active root.

reflexes, provides the only clue to the existence of membrane changes at the regions the threshold of which is tested.

The foregoing argument is based upon certain considerations of interaction between nerve fibers. If of two closely proximate groups of nerve fibers in a common conducting medium one group is active in the sense of conducting a volley of impulses, the other not, then: (a) fluctuations of membrane current occur in both groups of fibers (24); (b) threshold changes associated with the fluctuations of membrane current in the inactive fibers may be detected by means of suitable experimental procedure (24); but (c) an electrode placed in close proximity to the two groups of fibers (in association with an electrode elsewhere) records the membrane changes of the active fibers to the effective exclusion, at least, of those in the inactive fibers, unless and until the inactive fibers, by one means or another, as in "ephaptic" phenomena, themselves become active.

SUMMARY

The "dorsal root potential" consists of five successive deflections designated for convenience, D.R.I, II, III, IV, and V. Of these, D.R.V alone constitutes the dorsal root potential of prior description. A study has been made of the general properties of those deflections not previously described.

Dorsal root potentials are electrotonic extensions into the extramedullary root segment, the result of electrical interactions within the cord comparable to those that have been studied in peripheral nerve. Although the anatomical and electrical conditions of interaction are infinitely more complex in the cord than in nerve, it is seen that the fact of parallel distribution of primary afferent fibers pertaining to neighboring dorsal roots provides a sufficient anatomical basis for qualitative analysis in the first approximation of dorsal root potentials.

An extension of the theory of interaction between neighboring nerve fibers has been made to include an especial case of interaction between fibers oriented at right angles to one another. The predictions have been tested in a nerve model and found correct. Given this elaboration, and the stated anatomical propositions, existing knowledge of interaction provides an adequate theoretical basis for an elementary understanding of dorsal root potentials.

The study of general properties and the analysis of dorsal root potentials have led to the formulation of certain conclusions that follow.

D.R.I, II, and III record the electrotonic spread of polarization resulting from the external field of impulses conducted in the intramedullary segment and longitudinal tracts of primary afferent fibers.

D.R.IV arises in part as the result of activity in primary afferent fibers, and in part as the result of activity in secondary neurons. In either case the mode of production is the same, and the responsible agent is residual negativity in

the active collaterals, or, more precisely, the external field of current flow about the collaterals during the period of residual negativity.

Current flow about active primary afferent collaterals during the period of residual negativity is the agent for residual facilitation of monosynaptic reflex pathways. Since the changes in reflex threshold follow the course of residual negativity there is no need to postulate especial properties for prolonging action at regions the threshold of which is measured by means of monosynaptic test reflexes.

D.R.V results from polarization of primary afferent fibers by current flow about secondary neurons. There is indication that somata rather than axons of secondary neurons generate the polarizing currents. Similarity between D.R.V and the positive intermediary potential further indicates that soma gradients established during the recovery cycle are responsible for D.R.V.

Little or no net polarization of primary afferent fibers results from activity confined to the contralateral gray substance, the dorsal root potentials in contralateral recording resulting from interaction in the dorsal column or in the ipsilateral gray substance following decussation of contralaterally evoked activity.

During the course of asphyxia the initial defect in reflex pathways is the failure of secondary neurons to respond to primary impulses. Subsequently block is established at the branching zone of primary afferent fibers.

A relation exists between the sequence of dorsal root potentials and the cord potential sequence, the major departure from exact correspondence occurring in the region of D.R.IV and the negative intermediary potential and being of a nature to suggest that different aspects of internuncial activity are emphasized by the two methods of leading.

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STIMULATION OF TARSAL RECEPTORS OF THE BLOWFLY BY ALIPHATIC ALDEHYDES AND KETONES*

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In an effort to define the relationship between the chemical and physical properties of compounds and their effectiveness as stimuli for chemoreception, the response of blowflies (*Phormia regina* Meigen) to tarsal contact with aqueous solutions has now been studied in several different aliphatic series. Results obtained with the primary normal alcohols and with several types of glycols (2, 3) showed in each case a logarithmic increase in stimulative potency as the carbon chain was lengthened. Straight carbon chains proved to be more stimulating than isomeric branched chains or than chains containing ether linkages, and the simple alcohols were more effective than the corresponding dihydroxy compounds, a finding which agreed with results obtained earlier with various aliphatic acids (1). With the objective of gaining additional information as to the fundamental factors in sensory stimulation, we have in the present study extended this type of observation to two additional series, similar in chemical structure to those previously examined but with different polar groups.

Materials and Methods

Details of the experimental technique and of the method of treating the data have already been described (2, 3). Compounds used in the tests reported below included aldehydes, ketones, and a series of alcohols comparable to the latter in the position of the polar group. The following grades of chemicals were available:

Methanal.....	formaldehyde Merck reagent	
Ethanal.....	acetaldehyde Eastman	B. P. 20–22°C.
Propanal.....	propionaldehyde Eastman	B. P. 47–49°C.
Butanal.....	<i>n</i> -butyraldehyde Eastman	B. P. 72–74°C.
Heptanal.....	<i>n</i> -heptaldehyde Eastman	B. P. 40–42°C./10 mm.
2-Ethylhexanal.....	octaldehyde Carbon and Carbide Chemicals Corp.	B. P. 163.4°C.
2-Methylpropanal.....	<i>iso</i> -butyraldehyde Eastman	B. P. 63–64°C.

* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army and The Johns Hopkins University. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

3-Methylbutanal.....	<i>iso</i> -valeraldehyde Eastman	B. P. 91-93°C.
2-Propanone.....	acetone Merck reagent	
2-Butanone.....	methyl ethyl ketone Eastman	B. P. 79-80°C.
2-Pentanone.....	methyl <i>n</i> -propyl ketone Phillips Scientific Specialties Co.	
2-Heptanone.....	methyl <i>n</i> -amyl ketone Eastman	B. P. 149- 150°C.
3-Pentanone.....	diethyl ketone Eastman	B. P. 100.5-102.5°C.
4-Heptanone.....	di- <i>n</i> -propyl ketone Phillips Scientific Special- ties Co. (Technical grade).	
5-Nonanone.....	di- <i>n</i> -butyl ketone Eastman	B. P. 188.5- 192.5°C.
3-Pentanone, 2,4-dimethyl-.....	di- <i>iso</i> -propyl ketone Eastman (Pract.)	
2-Propanol.....	<i>iso</i> -propyl alcohol Eastman	98-99 per cent
2-Butanol.....	<i>sec</i> -butyl alcohol Eastman	B. P. 99-101°C.
2-Pentanol.....	<i>sec-act</i> -amyl alcohol Eastman	
2-Heptanol.....	methyl- <i>n</i> -amyl carbinol Paragon Testing Laboratories	
2-Octanol.....	capryl alcohol Merck	
3-Pentanol.....	diethyl carbinol Eastman	B. P. 114-116°C.

RESULTS

In Table I are collected the data required for a comparison of the compounds and for defining the response of the blowfly population to each.

DISCUSSION

Five normal aldehydes, two *iso*-aldehydes, and 2-ethylhexanal were tested. As shown in Fig. 1, the plot of rejection thresholds against chain length is similar in nearly all respects to that found previously for the normal alcohols, and includes a rather sharp break or change in slope between the three and four carbon members. The upper limb of the curve is fitted by a line having the equation

$$Y = 0.0690 - 1.2010(X - 0.2593)$$

(variance of $a = 0.0104$; variance of $b = 0.0232$)

and the lower limb (2-ethylhexanal excluded) by

$$Y = 1.9737 - 11.2533(X - 0.7153)$$

(variance of $a = 0.0176$; variance of $b = 1.2567$).

Although the slopes for these lines are not significantly different from those for the corresponding portions of the curve for normal alcohols (the difference in slope divided by its standard error is less than 3 in both cases), the position of the aldehyde curve is lower on the graph and the individual aldehydes were more stimulating than the corresponding alcohols in all of the seven pairs tested. They averaged about 2.66 times as effective.

Eight sufficiently water-soluble ketones were available. Since this small group includes representatives of several structurally different series and since some of the threshold values appear slightly aberrant, lines of best fit have not been determined. However, the ketones clearly follow the same sort of

TABLE I
Response of Phormia to Aldehydes, Ketones, and Secondary Alcohols in 0.1 M Sucrose

Compound	No. of C atoms	Log molar concentration rejected by 50 per cent ± 2.875 S.E.	$a \pm \text{S.E.}^*$	$b \pm \text{S.E.}^*$	\bar{x}^*	No. of flies tested
Methanal	1	0.365 ± 0.135	5.124 ± 0.144	2.766 ± 0.381	0.410	101
Ethanal	2	0.061 ± 0.147	4.467 ± 0.131	2.610 ± 0.350	-0.143	150
Propanal	3	-0.219 ± 0.120	4.952 ± 0.184	3.952 ± 0.718	-0.231	100
Butanal	4	-0.837 ± 0.147	4.858 ± 0.154	2.727 ± 0.440	-0.889	100
Heptanal	7	-3.531 ± 0.166	4.715 ± 0.145	2.371 ± 0.386	-3.651	100
2-Ethylhexanal	8	-3.688 ± 0.148	4.917 ± 0.155	2.709 ± 0.443	-3.718	100
2-Methylpropanal	4	-0.717 ± 0.152	5.159 ± 0.136	2.339 ± 0.336	-0.649	100
3-Methylbutanal	5	-1.553 ± 0.189	5.251 ± 0.173	2.449 ± 0.479	-1.450	75
2-Propanone	3	-0.094 ± 0.257	5.100 ± 0.200	2.022 ± 0.508	-0.044	135
2-Butanone	4	-0.597 ± 0.136	5.136 ± 0.133	2.540 ± 0.297	-0.544	120
2-Pentanone	5	-1.220 ± 0.162	5.010 ± 0.132	2.108 ± 0.277	-1.225	140
2-Heptanone	7	-2.529 ± 0.208	4.870 ± 0.208	2.600 ± 0.552	-2.579	105
3-Pentanone	5	-1.274 ± 0.145	5.183 ± 0.125	2.258 ± 0.310	-1.194	158
4-Heptanone	7	-2.855 ± 0.185	5.244 ± 0.156	2.789 ± 0.451	-2.768	100
5-Nonanone	9	-2.770 ± 0.171	4.900 ± 0.151	2.285 ± 0.418	-2.814	100
3-Pentanone, 2,4-dimethyl-	7	-2.022 ± 0.150	5.015 ± 0.144	2.471 ± 0.377	-2.020	100
2-Propanol	3	0.272 ± 0.036	5.244 ± 0.194	10.239 ± 1.725	0.296	105
2-Butanol	4	-0.011 ± 0.029	5.225 ± 0.154	11.300 ± 1.415	0.009	100
2-Pentanol	5	-0.856 ± 0.084	5.104 ± 0.155	4.753 ± 0.647	-0.834	124
2-Heptanol	7	-2.833 ± 0.161	5.008 ± 0.150	2.350 ± 0.394	-2.823	100
2-Octanol	8	-2.866 ± 0.193	4.950 ± 0.328	3.213 ± 0.766	-2.881	50
3-Pentanol	5	-0.908 ± 0.105	5.222 ± 0.111	2.801 ± 0.359	-0.829	160

S.E. = standard error.

* The 4th, 5th, and 6th columns of the table give the calculated values for a , b , and \bar{x} in the equation $Y = a + b(X - \bar{x})$, which is the regression of per cent flies rejecting, Y , expressed as probits, on log concentration, X .

relationship observed in other series and seem in general to be intermediate in stimulating effect between the corresponding alcohols and the aldehydes. That the ketones should be somewhat less effective than the aldehydes would be expected from the position of the polar group, which is terminal in the

aldehydes but subterminal in the ketones. As noted previously, alcohols with the hydroxy substitution elsewhere than in the 1-position are slightly less stimulating than their normal isomers (3). This is seen also in Fig. 1, where threshold values for five secondary alcohols and 3-pentanol may be compared both with the lines of best fit for the normal series and with the individual values for the corresponding ketones.

Altogether, the differences between any of the series, though fairly consistent,

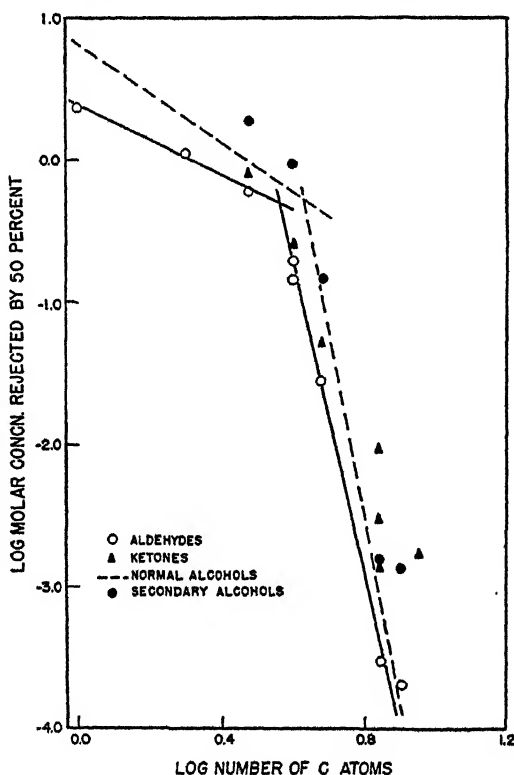


FIG. 1. Rejection of aldehydes, ketones, and secondary alcohols by *Phormia*.

are not great. It seems, therefore, that while the nature and position of the polar group are of some importance in determining the stimulating effect, the length of the carbon skeleton is a more significant factor for series of this sort.

The data now at hand for alcohols, glycols, aldehydes, and ketones suggest three principal questions: (1) Why is there regularly an increasing effectiveness of the members of each series as chain length is increased? (2) Why is the rate of increase in stimulating effect with increasing chain length less among the lower than among the higher members of a single series? (3) What chemical

or physical attributes determine the order of effectiveness of the several series studied, which is in descending order: aldehydes, ketones, alcohols, glycols?

Despite the fact that there is within each series a very high degree of correlation between the observed threshold values and boiling points or other properties related thermodynamically to them and to chain length, our attempts to find a single factor or set of factors which would reconcile the results from different series and from two portions of each series have been largely unsuccessful. The task has been made more difficult by the scarcity of pertinent physical data, for, with the exceptions of boiling and melting points, the values available for the various properties which might be significant are relatively incomplete, and in most cases a rigorous analysis has not been possible. Again, many of the properties themselves vary logarithmically with chain length and therefore yield correlations of the sort shown in Fig. 1, which does not bring the solution of the problem any nearer. Among these may be mentioned molecular weights, molecular volumes and areas, oil-water distribution coefficients, activity coefficients, standard free energies, vapor pressures, and boiling points.

Other objections to regarding the boiling point as an expression of the forces basically concerned in stimulation are the following: within a given series an increase in stimulating power accompanies an increase in boiling point, whereas the reverse is true when the several series are compared with one another. Thus, in the range of lower molecular weights, the order of increasing boiling points is: aldehydes, ketones, alcohols, diols; but this is the order of decreasing effectiveness in stimulation. Yet, among the various types of monohydric alcohols, boiling point and stimulating power both increase in the direction: secondary, *iso*-, primary. In view of contradictions such as these it seems certain that the factors involved in stimulation are not identical with those which determine the boiling point.

Dipole moments from the study by Smyth (8) gave an excellent correlation with threshold values in the series of normal alcohols (2). Possibly this was fortuitous, inasmuch as other determinations of dipole moments within a given series do not show a regular progression, and in fact vary but little (*cf.* reference 6). In any case, the differences between series cannot be explained on the basis of dipole moments, for the latter increase in passing from alcohols to aldehydes to ketones (4-6), and this is not the order of relative stimulating power. A plot of the threshold data against dielectric constants also was not particularly helpful, partly because of variation in the value for this property under differing conditions of measurement and the absence of standard conditions of measurement in many of the determinations reported.

Since it is known that the degree of association of polar-non-polar aliphatic compounds is greater in the lower range of molecular weights (9), it seemed possible that this might be concerned in the change in slope observed in plots of threshold values against chain length. Reasoning that the increase in boiling

point of the alkanes upon substitution of a polar group is in some degree a measure of the increased tendency for association (*cf.* reference 7), we compared the threshold values for the normal alcohols and aldehydes with the differences in boiling point between these compounds and the corresponding normal alkanes. However, this procedure failed to eliminate the change in slope. One may argue also that association would decrease the effective molarity of the test solutions; correcting for this, if possible, would merely decrease the observed threshold molarities more for the lower members of a series and would thus accentuate the difference in slope. For these reasons, it seems doubtful that degree of association is a factor of much importance in the observations.

We have been unable, then, to bring our data into a single homogeneous system through correlations with the following molecular properties: number of carbon atoms, molecular weights, molecular areas and volumes, oil-water distribution coefficients, activity coefficients, standard free energies, vapor pressures, boiling points, melting points, dipole moments, dielectric constants, and degree of association. Thus we must conclude either that we have overlooked the significant factors or that we are not dealing with a single process throughout, since the evidence from the series considered individually is convincing that stimulation is in some way related to the thermodynamic properties of the compounds.

One other comparison remains to be discussed. This is between the threshold concentrations of the compounds and their solubilities in water. Here again a quantitative evaluation is impossible, because of the incompleteness of published solubility data and because we have no means of grading the solubility among compounds which are freely miscible with water. It is a fact, however, that the order of stimulative effectiveness follows the inverse of the order of water solubilities with fewer contradictions than appear in most of the other comparisons attempted. Thus, within each series, solubility decreases consistently as chain length and stimulating power increase. The order of decreasing solubility: diols, alcohols, aldehydes is also the order of increasing stimulating effect, and *iso*-aldehydes are more soluble and less stimulating than the normal compounds, while the order of decreasing solubility for the isomeric alcohols: secondary, *iso*-, normal, is likewise that of increasing stimulation. 2-Butanone, on the other hand, is more stimulating than 2-butanol and slightly more soluble, so that it constitutes an exception; among the higher members compared in these two series both solubilities and threshold values tend to agree closely.

In spite of the generally good correspondence between low solubility in water and high stimulating power, it seems likely, from the data on oil-water distribution coefficients, that plots of threshold values against solubilities would also yield a smaller slope for the lower than for the higher range of compounds

in each series, if such an analysis could be made. The consistent recurrence of this type of relationship, which is well attested for the aldehydes, primary alcohols, and diols, strongly indicated for the ketones, *iso*-alcohols, and secondary alcohols (series lacking the one and two carbon members), and which seems to have no counterpart in any of the tabulated values for the physical properties, prompts us to consider the possibility that different forces may be of primary importance in stimulation by the lower and higher members of each of the types investigated. This amounts to postulating at least a two-phase system for the limiting mechanism in contact chemoreception. The hypothesis that smaller molecules gain access to the receptors in part through an aqueous phase, while the larger aliphatic molecules penetrate chiefly through (or accumulate in) a lipid phase, would appear to offer a basis for reconciling most of the contradictions encountered when it is attempted to fit the facts into a single-phase system. Movement of the smaller molecules through an aqueous medium should occur at rates related inversely to the molecular weight, which would help to account for their being more stimulating than anticipated from the relationship found for the higher members of a series, though it is doubtful that the entire difference can be explained in this way. It may be noted also that the inflections in the curves relating thresholds to molecular size occur at increasing chain lengths in passing from the less to the more water-soluble species. At the same time the predominant importance of lipid affinity is suggested by the logarithmically increasing stimulating power of both lower and higher members of all series, as well as by the inverse relationship between water solubility and stimulating effectiveness in comparisons of the several series with each other. Although it is not known to what extent recent observations on the structure of insect cuticle (10, 11) may apply to the chemoreceptive surface, a scheme such as that proposed here is in accord with current views as to the general makeup of the integument, and would also allow for the fact that highly water-soluble ions as well as nearly insoluble non-electrolytes are adequate stimuli when presented in an aqueous medium.

SUMMARY

Rejection of eight aldehydes, eight ketones, five secondary alcohols, and 3-pentanol has been studied in the blowfly *Phormia regina* Meigen. The data agree with results previously reported for normal alcohols and several series of glycols in showing a logarithmic increase in stimulating effect with increasing chain length. The order of increasing effectiveness among the different species of compounds thus far investigated is the following: polyglycols, diols, secondary alcohols, *iso*-alcohols, normal alcohols, ketones, *iso*-aldehydes, normal aldehydes.

Curves relating the logarithms of threshold concentration to the logarithms of chain length for diols, alcohols, aldehydes, and ketones show inflections in

the 3 to 6 carbon range. Above and below the region of inflection the curves are nearly rectilinear. The slopes for the upper limbs (smaller molecules) are of the order of -2 ; for the lower limbs, about -10 .

Comparisons of the threshold data with numerical values for molecular weights, molecular areas and volumes, oil-water distribution coefficients, activity coefficients, standard free energies, vapor pressures, boiling points, melting points, dipole moments, dielectric constants, and degree of association are discussed briefly, and it is concluded that none of the comparisons serves to bring the data from the several series and from the two portions of each series into a single homogeneous system. A qualitative comparison with water solubilities shows fewer discrepancies.

It is suggested that the existence of a combination of aqueous and lipid phases at the receptor surface would fit best with what is presently known about the relationship between chemical structure and stimulating effect in contact chemoreception. In this hypothesis the smaller and more highly water-soluble compounds are envisaged as gaining access to the receptors partly through the aqueous phase, the larger molecules predominantly through the lipid phase.

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THE ELECTRICAL PROPERTIES OF SYNTHETIC MEMBRANES

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INTRODUCTION

The purpose of this paper is to present the results of a preliminary investigation of the electrical properties of synthetic membranes which are strong electrolytes. Cole (1) has studied a variety of biological membranes and found that their impedance locus (2) is, in general, a circular arc. Michaelis (3) and his coworkers have made extensive studies of collodion membranes; likewise, Sollner (4) has worked with a number of synthetic membranes, especially those derived from cellulose. But most of this work was primarily concerned with concentration potentials and osmotic behavior. Goldman (5), however, studied the A. C. electrical properties of collodion membranes containing phospholipids, and found impedance plots similar to those found by Cole for the biological membranes.

Working with naturally occurring substances involves several inherent difficulties: the structure is not always known; the structure is determined by the source of the material and not by the experimenter; and many of the materials (especially proteins and living membranes) change chemically and physically on standing. In connection with Project NR 054-002 of the Office of Naval Research, we have recently synthesized some polyelectrolytic materials which can be cast in the form of thin films. Some of these are insoluble in water. In contrast to most natural substances, which are either weak carboxylic acids or nitrogen bases, our compounds are strong electrolytes, similar electrically to potassium bromide. It therefore seemed of interest to investigate their electrical properties; although this work was interrupted at an early stage, some results were obtained which may be useful.

EXPERIMENTAL

A mixture of 5 parts 4-vinylpyridine and 95 parts styrene were copolymerized (6) in 20 per cent solution in toluene, using 1 per cent benzoyl peroxide on the monomer mixture as a catalyst. The resulting product is a linear polymer which has on an average a pyridyl group attached to every fortieth carbon atom of the chain. By addition of *n*-butyl bromide in nitromethane solution to the copolymer, the heterocyclic nitrogens are converted to butyl-pyridonium ions

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and the resulting structure is now a polyelectrolyte, with large cations and small, mobile counter ions. Solutions of the polyelectrolyte in methyl ethyl ketone gave by our usual casting technique (7) films which were much too brittle to handle. On plasticizing with 30 per cent by weight of dutrex 25 (Shell Oil Company; suggested by Mr. Robert L. Speer), films were obtained which were pliable when warm.

These films were clamped between the halves of a split conductance cell (internal diameter, 22 mm.) with platinized disc electrodes about 40 mm. to each side of the membrane. The cell was then filled with electrolyte and the composite series circuit

Electrolyte / Membrane / Electrolyte (I)

was measured at various frequencies in the audio-range, using a General Radio type 716 C bridge. The cell was placed in a metal box for electrical shielding; shield and one electrode were connected to the grounded point of the bridge and the other electrode to the "unknown" terminal. For more precise work, a guarded circuit should, of course, be used. Correction was made for lead and stray capacity. Measurements were made at room temperature (*ca.* 26°); our cell design was not suitable for thermostating.

Bridge balance drifted very rapidly immediately after filling the cell with electrolyte or after changing electrolyte, but gradually settled down to reasonable constancy after about 24 hours. These drifts were largely due to the diffusion involved in setting up equilibrium between the ions in the solution and those in the membrane. As a matter of fact, the membrane acts much like an exchange resin; the polycations are immobilized in the membrane while the anions are free to diffuse into and through it.

RESULTS

Results for a typical membrane in 0.1 *N* potassium bromide are shown in Table I, where frequency, (observed) equivalent parallel capacitance, and (observed) tangent of loss angle are given in the first three columns. The average thickness of the membrane, computed from weight (0.173 gm.) and area (3.8 cm.²) was 0.0140 cm.; the equivalent geometrical capacitance C_0 of the membrane was 24.4 μf . The resistance of the cell, filled with 0.1 *N* potassium bromide and without the membrane barrier, was 177 ohms; since the resistance of the aggregate (I) was of the order of many megohms, we may neglect the resistance of the electrolyte used to make contact with the membrane and assume the capacity and tangent of Table I to refer to the membrane alone.

If we consider the membrane and the electrolyte it contains as a parallel circuit, the resistance element R_x is given from the observed data by the familiar relationship

$$\tan \delta = 1/\omega C_x R_x.$$

Cole and his coworkers usually convert their data to the equivalent *series* circuit by means of the equations

$$R_s = \frac{R_x}{1 + (R_x C_x \omega)^2} = \frac{1}{\omega C_x} \frac{\tan \delta}{1 + \tan^2 \delta}$$

$$-X_s = \frac{R_x^2 C_x \omega}{1 + (R_x C_x \omega)^2} = \frac{1}{\omega C_x} \frac{1}{1 + \tan^2 \delta}$$

A plot of $(-X_s)$ against R_s gives a circular arc. We have computed the observed values of C_x and $\tan \delta$ of Table I to the corresponding equivalent series quantities; these data are shown in Fig. 1. It will be seen that the points lie on a curve, which over its somewhat limited range can be fitted by a circular arc

TABLE I
*Electrical Properties of Membrane of Copolymer 4-Vinyl-N-n-Butyl Pyridonium
Bromide-Styrene*

f	C_x	$\tan \delta$	ϵ''	ϵ'
100 c.	342.0 μmf	0.427	0.94	(14.02)
150 "	308.8	0.334	0.84	(12.58)
200 "	292.0	0.280	0.81	(11.97)
400 "	266.4	0.196	0.86	(10.92)
500 "	260.5	0.175	0.85	(10.58)
1 kc.	242.0	0.126	0.74	9.91
2 "	232.6	0.099	0.69	9.55
5 "	223.6	0.075	0.59	9.15
10 "	216.0	0.064	0.52	8.85
20 "	211.0	0.058	0.48	8.65

whose center lies just below the horizontal axis. The extrapolated limit corresponding to zero frequency is 13.0 megohms, which agrees with the value found by another extrapolation method which will be described shortly. Our lowest frequency, 100 cycles, carries us only about one-third the way around the arc; it is obvious that very low frequencies would be needed to reach the descending limb of the curve for our membranes. The similarity to the curves for natural membranes is, however, unmistakable.

It seems reasonable to assume that part of the in-phase current through the membrane is purely electrolytic, and part of it is an A. C. loss associated with a relaxation process. This suggests another analysis of the data. Suppose we calculate the electrolytic loss, subtract this from the total in-phase component, and then examine the resulting admittance which will be the response due to the membrane itself. A specific conductance κ_0 is equivalent (8) to an ionic loss factor ϵ''_i ; at frequency f given by

$$\epsilon''_i = 9\kappa_0 \times 10^{12}/5f. \quad (1)$$

The total loss factor observed, ϵ''_t , is then

$$\epsilon''_t = \epsilon'' + \epsilon''_i \quad (2)$$

where ϵ'' is the power absorption per cycle due to A. C. mechanisms such as dipole rotation. If we recall that

$$\tan \delta = \epsilon''_t / \epsilon' = \epsilon''_t C_0 / C_x \quad (3)$$

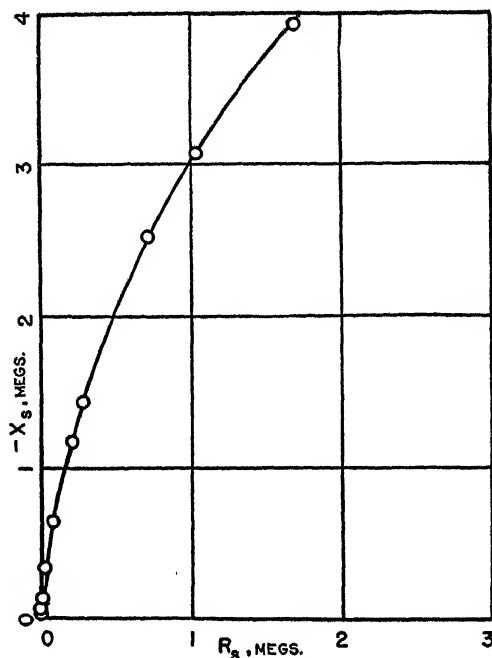


FIG. 1. Cole plot for synthetic membrane.

where C_x is the measured unknown capacity and ϵ' is dielectric constant, we have, on substitution of (1) and (3) into (2)

$$f C_x \tan \delta = 1.8 \times 10^{12} C_0 \kappa_0 + \epsilon'' C_0 f \quad (4)$$

Equation (4) furnishes a convenient means of analyzing the data to separate A. C. and D. C. components, because ϵ'' does not, as a rule, change rapidly with frequency at low frequencies for polymeric systems (9). As shown in Fig. 2, a plot of $f C_x \tan \delta$ against frequency is linear and extrapolation to zero frequency determines the ionic conductance κ_0 :

$$\kappa_0 = (f C_x \tan \delta)_{f=0} / 1.8 \times 10^{12} C_0 \quad (5)$$

where $(f C_x \tan \delta)_{f=0}$ is the intercept I at zero frequency. For the example of Table I and Fig. 2, we find $\kappa_0 = 2.8 \times 10^{-10}$ mho. Membranes were

measured in other electrolytes, including hydrobromic acid and potassium hydroxide solutions; very briefly summarized, the D. C. conductance of the membrane changed with the electrolytic environment, *but the A. C. properties were substantially unaffected.*

The difference between $C_x \tan \delta$ and the ratio of intercept to frequency then gives the A. C. loss factor after division by the geometrical capacitance C_0 :

$$\epsilon'' = (C_x \tan \delta - I/f)/C_0 \quad (6)$$

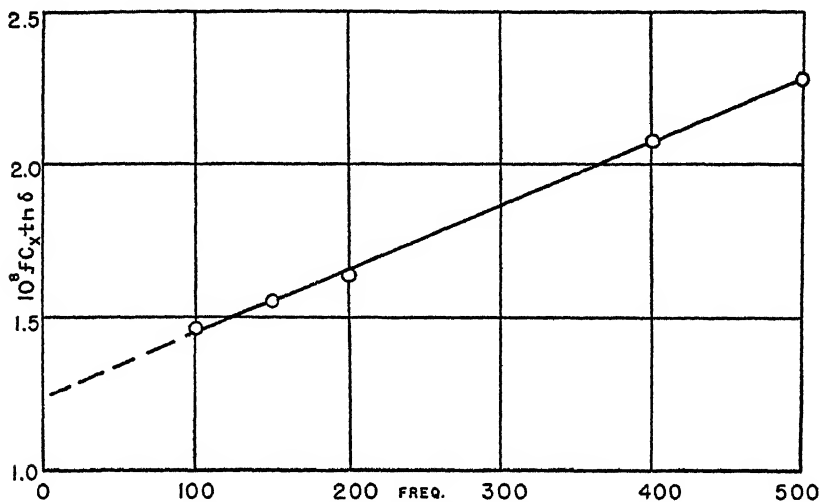


FIG. 2. Determination of D. C. conductance.

These values are given in the fourth column of Table I; the dielectric constants,

$$\epsilon' = C_x/C_0 \quad (7)$$

are given in the fifth.

A plot of the loss factors *versus* logarithm of frequency is given as the middle curve of Fig. 3 (ordinates right); the data at low frequencies scatter somewhat, because they represent a small difference between large quantities (*cf.* Fig. 2) but a maximum in the decade between 10 and 100 cycles is indicated. The very slow decrement in the audio-frequency range suggests the presence of a wide distribution (10) of relaxation times in the membrane. When dielectric constant is plotted against $\log f$, an approximately linear curve is obtained in the audio-frequency range, which is again characteristic of a broad distribution. A sharp rise in apparent dielectric constant is noted below 1 kilocycle; we are inclined to ascribe this to electrode polarization and to assume that the dielectric constant of the membrane follows the solid curve in Fig. 3. (Similar fictitious sharp rises in dielectric constant have been observed for other systems (11) at low frequencies.)

In order to compare the data with those for other systems, we used the method of Fuoss and Kirkwood (10): a plot of hyperbolic anticosine of the ratio (ϵ''_m/ϵ'') against logarithm of frequency is shown as the lower curve of Fig. 3. For ϵ''_m , the maximum value of the A. C. loss factor, we used 0.90. The audio-frequency points lie on a straight line with slope 0.475; multiplica-

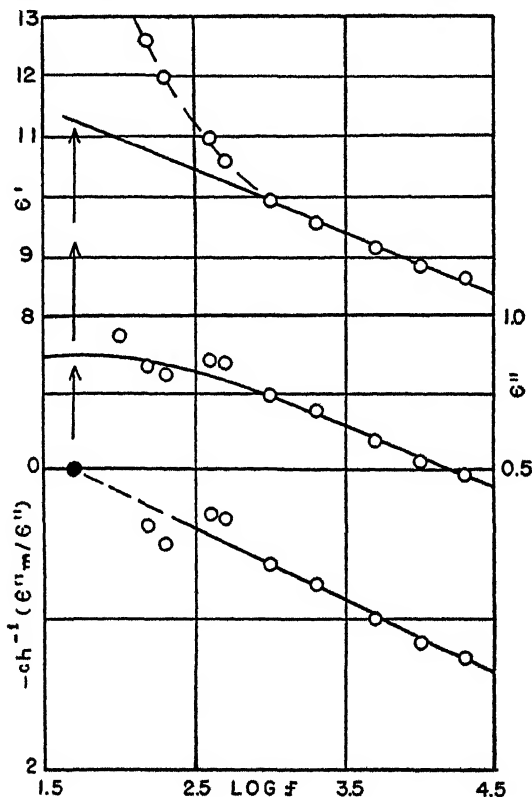


FIG. 3. Membrane characteristics.

tion by 0.4343 gives 0.206 as the value of α , the distribution parameter (10). Extrapolation of the audio-frequency (12) data to $ch^{-1}(\epsilon''_m/\epsilon'') = 0$ determines $\log f_m = 1.70$; *i. e.*, the peak loss is at 50 cycles. Using Table VII of reference 12, we find that $\alpha = 0.206$ corresponds to $\beta = 0.284$. By extrapolating the solid curve at the top of Fig. 2 to $\log f = 1.70$, we find $\epsilon'_m = 11.5$ as the dielectric constant of the membrane at the frequency corresponding to maximum loss. Using equations (19) and (20) of reference 12, we find $\epsilon_0 = 15.5$ as the static dielectric constant and $\epsilon_\infty = 7.5$ as the limiting dielectric constant of the membrane. The latter value is much higher than the probable index of re-

fraction; it seems pointless to discuss this item until further data are available, because our value of C_0 for example, may, be incorrect.

From the values of β , ϵ'_m , and ϵ''_m , we may, however, compute the constants of a circular arc which is shown as the solid curve in Fig. 4. With the exception of the 100 cycle point, the data conform very well to this arc.

The results show that the synthetic membrane gives an impedance locus which is similar to that found for biological membranes; furthermore, we can separate the total electrical response into an in-phase electrolytic conductance superimposed on a purely A. C. mechanism. This suggests that a similar interpretation might be applied to the data on the natural membranes. If we had a pure capacity parallel with an electrolytic resistance, the $X_c - R_c$ plots would

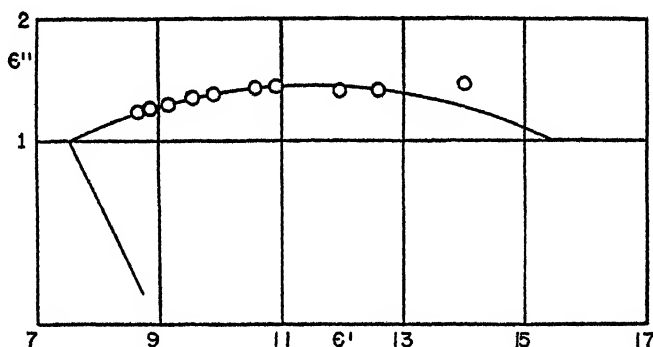


FIG. 4. A. C. components of synthetic membrane.

be true semicircles; as A. C. loss develops in the capacitive component, the center of the arc drops below the horizontal axis. But regardless of the properties of the capacitor, the center will always be near the axis when the parallel resistance is low; in other words, a high electrolytic conductance can mask the properties inherent in the structure of the membrane itself. In our membranes, for example, the broad distribution of relaxation times characteristic of the polymer structure is revealed only after correction is made for the electrolytic conductance.

In any case, the data clearly show that synthetic membranes which contain polar groups, and which permit ion exchange, give A. C. electrical properties which are similar in a general way to those exhibited by biological membranes. As suggested in the introduction, it does seem that the synthetic membranes offer another tool in biological research, because, within reasonable limits, their structure and environment may be varied at will. Analogies in electrical properties between natural and synthetic membranes may then permit some conclusions about the structure of the former.

SUMMARY

By the addition of *n*-butyl bromide to a 1:19 copolymer of 4-vinylpyridine and styrene, water-insoluble, strong polyelectrolytes can be prepared. The addition of a hydrocarbon plasticizer permits the casting of flexible films in which large polycations are immobilized but in which bromide ions (or other small anions) are free to move. Electrical measurements on these membranes showed that they could be represented by a complex admittance: an electrolytic conductance in parallel with a pure A. C. impedance. The latter gives a circular arc when real component is plotted against imaginary. These synthetic membranes thus resemble in their electrical behavior that found by Cole for a variety of biological membranes.

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THE RATE OF LOSS OF POTASSIUM FROM HUMAN RED CELLS IN SYSTEMS TO WHICH LYSINS HAVE NOT BEEN ADDED

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Prolytic cation exchanges have now been described in a variety of systems containing human red cells and lysins in hypolytic concentration (Ponder, 1947 *a, b, c*; 1948 *a, b*), and an Na-K exchange has also been observed to occur when the cells are suspended in isotonic or hypotonic NaCl containing no added hemolysins. In systems containing lysins, and particularly when the prolytic losses are large, the loss of K increases with time until the K concentration inside the cell is approximately the same as that in the medium outside; *i.e.*, the exponential function of time which describes the K loss approaches the line $K = 1.0$ as an asymptote (Ponder, 1948 *b*). The course of the K loss (and Na gain) in systems containing no added lysin, on the other hand, has not been so clearly established. There are very definite indications (Davson, 1937, Rapoport, 1947) that the losses are rapid at first and that they slow down so that a new steady state, remote from the equilibrium state at which $K = 1.0$, is reached; most of the experiments which point to this conclusion, however, are of short duration, and one of the points which has been brought out by the investigations of prolytic ion exchanges is that the course of the curves which relate ion exchanges to time can be ascertained only by experiments which extend over long times. If the curve for the time-course of K losses in systems containing some lysins is different from that for the time-course of K loss into lysin-free NaCl, in that the latter terminates in a new steady state while the former ends in an equilibrium, it is not certain that the time-course of the ion exchanges is not of an intermediate form in systems containing other lysins or the same lysins in smaller concentrations. Many relations are possible, and those which are characteristic of a given system require to be found by systematic exploration.

This paper is concerned with the results obtained in four kinds of system: (1) washed red cells in saline at 4°C., (2) washed red cells in saline at 25°C., (3) washed red cells in saline at 37°C., and (4) washed red cells in systems, at 4°C., 25°C., and 37°C., containing hypotonic saline, glucose, or a number of other substances the addition of which throws light on the nature of the phenomena observed. A consideration of what happens in these four types of system is sufficient to outline the principal aspects of the problem. It should be pointed out, however, that the purpose of this investigation is to describe the types of relation found between the rate of K loss and time rather than to answer the

many questions arising in connection with the forms which the relations take under different conditions.

Methods

It is essential that the extended observations of the rate of K loss from the red cells be carried out under aseptic conditions, since contamination of the system by bacteria may be tantamount to the addition of a hemolysin. A satisfactory procedure is one based on the methods used by Osgood (1939) for marrow culture. All manipulations are carried out with syringes and in 15 ml. tubes covered with vaccine caps. The caps are inserted in the tubes before sterilization, and the blood, washing fluids, etc., are introduced with sterile syringes and needles after wiping the cap with 95 per cent alcohol and inserting an air vent consisting of a needle connected by sterile rubber tubing to an air filter. The possibilities of contamination can be reduced by packing each syringe, etc., in its own pack, and by sterilizing each needle in a separate rubber-capped glass tube; by suitable arrangement of the equipment so as to constitute almost a closed system, it is possible to work without contamination in an ordinary laboratory room. The various saline solutions are sterilized in vaccine-stoppered flasks fitted with air vents and air filters.

The blood is drawn from healthy donors into sterile flasks containing a few drops of liquamin (a preparation of heparin for intravenous use). Almost all the systems with which this investigation is concerned are composed of washed cells added to saline,¹ saline plus glucose, etc., and are made by adding 2 ml. of a cell suspension of volume concentration 0.4 to 10 ml. of saline, saline plus glucose, etc. Since a small quantity of the system is needed for the preparation of the standards representing complete hemolysis and complete K loss, a 2 ml. sample can be withdrawn at only five intervals of time from each system of 12 ml. volume; these intervals require to be suitably spaced as a result of experience.

The completed systems are kept mixed by inverting the tubes containing them every 1 to 2 hours, and are oxygenated by drawing sterile air through an air filter as the withdrawals are made. These withdrawals are made with 2 ml. syringes, a needle connected with an air filter being inserted as an air vent. The systems are tested from time to time for their sterility. When determinations are made at 37°C. and also in some experiments at lower temperatures, the systems are contained in small rubber-capped Erlenmeyer flasks attached to a shaking apparatus in a constant temperature bath. By using long needles inserted through the rubber caps, sterile air or gas mixtures moistened by bubbling through warm saline can be pumped through the systems. Samples are withdrawn at intervals, as already described.

Washed cells are used in most systems, the washing being carried out at room tem-

¹ The suspension medium may be either freshly prepared NaCl (172 m. eq./litre) or a NaCl-buffer composed of a mixture of NaCl in a concentration of 10 gm./litre and various proportions of M/15 NaH_2PO_4 and M/15 Na_2HPO_4 . The effect of adding buffer and controlling the pH within the range 6.0 to 7.5 is quite small, the K losses being only about 10 per cent smaller in a system buffered at pH 7.5 than they are in freshly prepared unbuffered NaCl (pH 6.0 to 6.5).

perature (25°C.). A small sample of the completed system is set aside for K analysis. This step is important, for considerable amounts of K may be lost during the washing, etc., and the initial value for the concentration gradient of K may be considerably lower in the case of washed red cells than in the case of unwashed ones. When unwashed cells are added to complete the systems, small volumes of the latter are set aside in a similar way; the cells are spun down, packed, and their K content is determined.

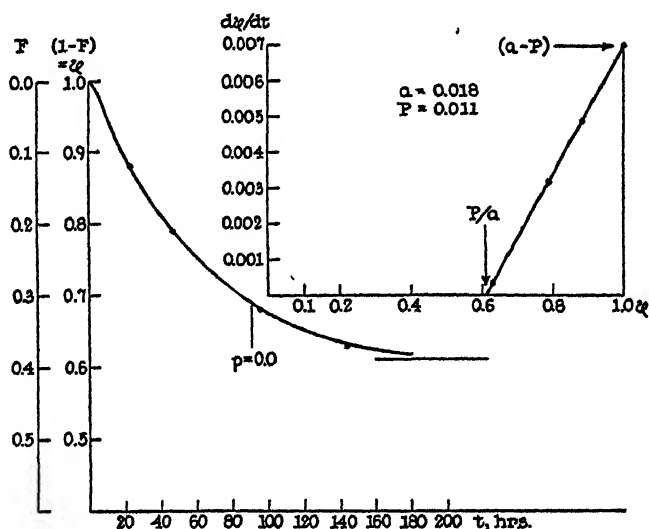


FIG. 1. Loss of K from washed human red cells into 172 m. eq./litre NaCl at 4°C. Ordinates, fraction of initial K lost from intact cells, F ; ϕ or $(1 - F)$. Abscissa, time in hours. The point $p = 0.0$ marks the beginning of hemolysis. Inset, analysis of the curve in terms of P and a .

1. K. Losses at 4°C.

A typical curve relating the loss of K from washed human red cells into 172 m. eq./litre NaCl at 4°C. is shown in Fig. 1. The experimental values of K, the K lost expressed as a fraction of the initial K content of the cells K_0 , are given in Table I together with the amount of lysis in the system ($p = 1.0$ for complete hemolysis). From the values of K and of p , the fraction of their initial K which has been lost by the intact cells of the system can be calculated: it is

$$F = (K - p)/(1 - p),$$

and the fraction of their initial K which the intact cells of the system still contain is $(1 - F)$. The values of $(1 - F)$ decrease with time, in such a way as to suggest that a new steady state with a value of $(1 - F)$ in the neighborhood of 0.6 is reached after very long times. There are several possible ways in which such a steady state might be reached and maintained (Ponder, 1948 a);

the cells may have a membrane which goes through a phase of permeability to K, and then becomes impermeable to K again, there may be an active ion transfer mechanism which regulates the distribution of ions such as K and Na, or the ionic distribution may be maintained by "ion-binding" processes involving the material in the cell interior rather than by a permeability process as ordinarily understood. The observations of Davson (1937) on the loss of K into hypotonic media constitute the principal experimental evidence (apart from the shape of the curve relating K loss to time) in favor of the possibility that a membrane undergoes a transient phase of increased permeability; the observations themselves and the conclusions drawn from them, however, are questionable (see section 4, below), and do not form a satisfactory basis for a theoretical treatment. The last possibility is also unsuitable as a starting point for a general formulation because it is too vague; this leaves for consideration

TABLE I
Relation of K Loss to Time at 4°C.

<i>t</i>	K	<i>p</i>	<i>F</i>
<i>hrs.</i>			
24	0.12	0.00	0.12
48	0.21	0.00	0.21
96	0.32	0.02	0.31
144	0.37	0.04	0.37

the possibility that the loss of K and the attainment of the steady state are due to changes in the activity of an ion transfer process.

Suppose that $(1 - F)$ or ϕ , the quantity of K which the red cell contains, is determined by a process which causes K to accumulate at a rate P , and that K tends to leave the cell at a rate Q because the cell is permeable to it. For the time being, also suppose that the concentration of K in the medium surrounding the cell is small as compared with the concentration of cell K. The rate at which K leaves the cell will then depend on ϕ itself and on a diffusion constant a which may have the special meaning of a permeability constant and which may vary explicitly with time. The net rate of decrease $-d\phi/dt$ is the difference between the rate of accumulation and the rate of escape,

$$-d\phi/dt = P - a\phi \quad (1)$$

To determine a , draw tangents to the experimental curve and plot their slopes $d\phi/dt$ against ϕ (inset of Fig. 1). The result in this case is a straight line with a slope of $a = 0.018$, making an intercept $(a - P) = 0.007$ on the ordinate at $\phi = 1.0$. The value of P is accordingly 0.011. The line also makes an intercept on the $\phi -$ axis where $\phi = 0.61$. This gives the position of the

asymptote, $\varphi_{\infty} = 0.61$, which the experimental curve approaches at infinite time.²

In sterile systems containing human red cells at 4°C., this type of curve is obtained consistently, and the disturbing possibility that the apparent attainment of a new steady state is due to the restriction of the period of observation (Ponder, 1948 *b*) seems to be disposed of. Table II gives values of φ_{∞} , a , and P

TABLE II
Values of φ_{∞} , a , and P at 4°C.

Donor	φ_{∞}	a	\dot{p} at 144 hrs.	P
1	0.49	0.0095	0.08	0.0047
2	0.37	0.0054	0.08	0.0020
3	0.56	0.0091	0.04	0.0052
4	0.58	0.0097	0.03	0.0056
5	0.61	0.0180	0.04	0.0110
6	0.65	0.0140	0.07	0.0091
7	0.50	0.0100	0.06	0.0050
8	0.60	0.0110	0.04	0.0066
9	0.51	0.0092	0.04	0.0047
10	0.50	0.0090	0.05	0.0045
Average.....				0.0058

found in a series of observations at 4°C. on systems containing the cells of different donors.

The K losses shown in Fig. 1 were determined at intervals of 24, 48, 96, and 144 hours after the beginning of the experiment, so as to define as much of the course of the curve as possible by the position of four points. As in the case of the curves obtained in experiments on systems in which lysins were present initially (Ponder, 1948 *b*), the course of the K loss during the first few hours is therefore left undefined. These losses are very small in systems at 4°C., and it is only at higher temperatures, and even then only under special circumstances, that they become easily measured; the detailed consideration of the K losses occurring during the first few hours is accordingly better left until the events in systems at 37°C. are described. In the meantime, it is sufficient to say that at time $t = 0$ the cells of the system are apparently

² The solution of the differential equation (1) when a is a constant is

$$\varphi = (1 - P/a) e^{-at} + P/a$$

so φ_{∞} also equals P/a . The value of $(1 - \varphi_{\infty})$ is the same as that denoted by K_{∞} in the expression

$$dK/dt = k(K_{\infty} - K)$$

which describes the experimental curve from the standpoint of the amount of K lost by the cell and found in the surrounding medium (Ponder, 1948 *a*, expression 1).

in a steady state (approximating to the steady state which they would have maintained in the blood stream if left there), and that the value $\varphi = 1.0$ is itself equal to P/a_0 , where P is the rate of accumulation (supposed to remain constant) and a_0 a constant regulating the rate of escape characteristic of the cells in their "normal" state in plasma in which the net escape of K is zero. What we are assuming to happen is that a increases so that K is lost and a new steady state defined by φ_∞ is reached. If the change in a is not an instantaneous one, the transition from one steady state to another occurs along a sigmoid path (Ponder, 1944), and the course of the relation between

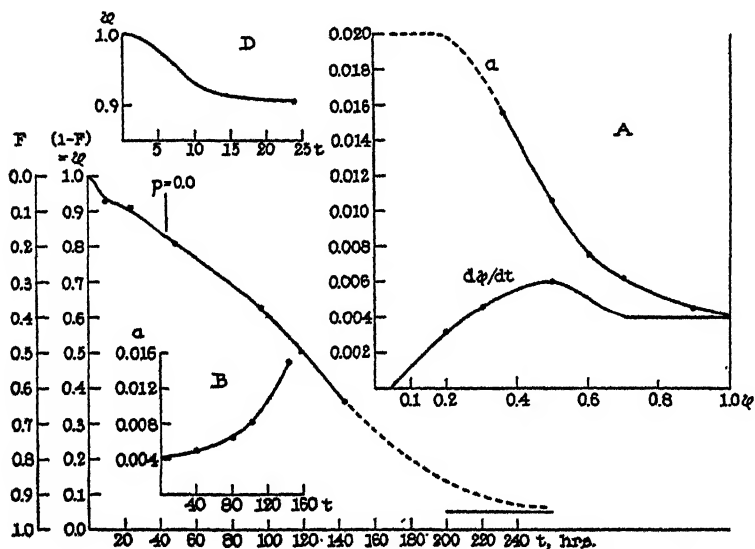


FIG. 2. Loss of K from washed human red cells into 172 m. eq./litre NaCl at 25°C. Ordinates and abscissa as in Fig. 1. Inset A, values of a , P being assumed to be zero. Inset B, values of a as a function of time. Inset D, the initial part of the curve shown on an enlarged scale.

φ and t during the first 6 to 12 hours is probably always similar to that shown in Fig. 2, inset D (*cf.* Ponder, 1947 *b*, footnote 3).

This analysis is based on the assumption that the rate of K loss from the red cell is due to a change in the value of a , the constant which regulates the rate of diffusion of K from the inside of the cell to the outside. Since what is measured is $d\varphi/dt$, the difference between P and $a\varphi$, it is equally possible that the losses of K are due to P varying explicitly with time; the same experimental curve would result, for example, from P suddenly changing from P_0 at $t = 0$ to $0.61P_0$, a remaining constant. Further, both a and P might vary with time. This analysis does not distinguish between these possibilities, its purpose being to provide a method, somewhat non-committal in the meantime, of dealing with the experimental observations. The possibility that P varies with time will

require to be considered when the effect of agents which alter metabolism, such as NaF and NaCN, is described (see section 4, below).

2. *K Losses at 25°C.*

A typical curve for the rate of the loss of K from human red cells into 172 m. eq./liter NaCl at 25°C. is shown in Fig. 2. This curve is plotted from experimental data (Table III) treated in exactly the same way as those from which the curve of Fig. 1 is constructed. Unlike the latter, it shows no indication of reaching any steady state other than that at which the K concentration inside and outside the cell would be equal. If the course of the K loss during the first few hours is left unconsidered in the meantime, the curve proceeds down-

TABLE III
Relation of K Loss to Time at 25°C.

<i>t</i>	K	<i>p</i>	<i>P</i>
<i>hrs.</i>			
24	0.09	0.00	0.09
48	0.19	0.01	0.18
96	0.40	0.05	0.37
144	0.68	0.12	0.64

wards almost linearly from the origin at $\varphi = 1.0$, $t = 0$ until the last experimental point is reached at $\varphi = 0.36$, $t = 144$.

Beyond this, good experimental values cannot be obtained because of the increasing amount of hemolysis. The probable course of the curve can be conjectured, however, by analogy with the course of curves for systems containing lysins (Ponder, 1948 *b*), and is shown in Fig. 2 as the dotted portion of the curve approaching an asymptote in the neighborhood of $\varphi = 0.05$; at this value of φ , the K concentrations inside and outside the cells would be equal.³ *P* can accordingly be supposed to be equal to zero in the expression

$$a = \frac{P + d\varphi/dt}{\varphi} \quad (2)$$

and *a* can be evaluated on the assumption that the final value of *P* applies to the whole curve.⁴ The result is shown in inset A of Fig. 2. The value of

³ The value of φ at which the K concentrations inside and outside the cell are equal is approximately v/V , where *v* is the volume of the fluid phase in the cells and where *V* is the volume of the surrounding medium. In these systems $v = 0.56$ ml. and $V = 11.76$ ml., so $\varphi_{\infty} = 0.047$. If *V* were infinitely large, φ_{∞} would be zero.

⁴ It is quite likely that *P* is zero only when *t* is great and that it is positive when *t* is smaller. If this were so, the values of *a* would be larger than those calculated on the basis of *P* being zero; i.e., the values of *a* shown in the inset of Fig. 2 are minimum

$d\varphi/dt$ remains comparatively constant as φ decreases, passes through a maximum, and then decreases; the value of a increases from about 0.004 at $\varphi = 1.0$ to about 0.020 at $\varphi = 0.05$.

Since φ decreases with time and a increases as φ decreases, a can also be shown as a function of time (Fig. 2, inset B). This way of looking at the situation suggests that the difference between the type of relation found at 4°C. and that found at 25°C. is that the constant a , which is equivalent to a permeability constant in the way in which it regulates the rate of escape of K from the cells

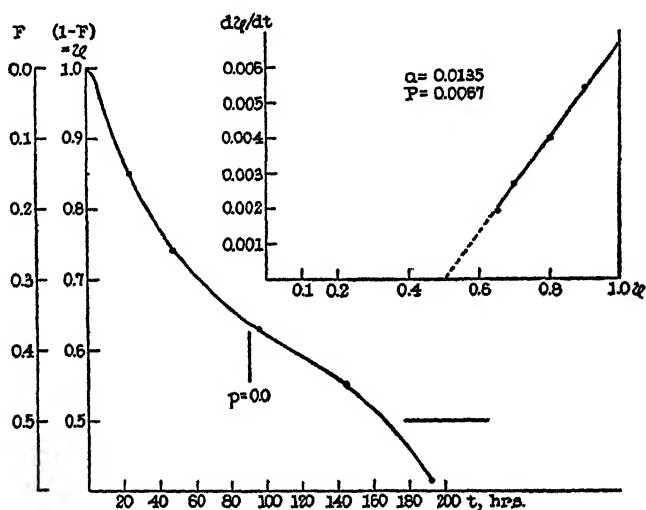


FIG. 3. An "intermediate" type of curve, obtained at 4°C. Ordinates and abscissa as in Fig. 1. Inset, analysis as in Fig. 1.

of the system, increases with time in systems at 25°C. instead of remaining substantially constant as it does at 4°C. Taking into consideration that the systems at 25°C. show much more hemolysis than corresponding systems at 4°C. do, the most likely cause of the continuous increase in a observed at 25°C. is an autolytic process. The effect of this reaction seems to be (a) to increase the value of a as time progresses, and (b) to decrease the value of P from its initial value to zero, probably as a function of time.⁵

values. In the meantime there is no way of examining this possibility further; what is needed is an independent method for finding P (or a) as a function of φ or of t . Studies of red cell metabolism might conceivably provide the necessary information. Until this is forthcoming, the experimental curves can be analyzed only in a qualitative sort of way.

⁵ In the case of systems containing lysins such as resorcinol, the curve relating the loss of K to time is one for which P is zero, or nearly zero, and a constant and large,

While the assumption that the value $P = 0$ applies to the entire curve can be made for the purpose of discussing the probable nature of the relations, it is not tenable for the purpose of an analysis, in unequivocal terms, of the curves obtained at 25°C. (see footnotes 3, 4, 6).

Intermediate Types of Curve.—The conclusion that the curve relating φ and t at 4°C. is an essentially different type of curve from that obtained at 25°C suggests that there may be curves which are intermediate in type. These are sometimes met with in experiment, and one of them is illustrated in Fig. 3. This curve (human red cells 172 m. eq./liter NaCl at 4°C.) starts off as if proceeding to an asymptote at $\varphi = 0.50$ ($a = 0.0135$, see inset of Fig. 3). After 100 hours, the curve bends downwards and there is little doubt but that its further course is similar to that of the curve shown in Fig. 2. The simplest explanation of this situation is that there is negligible autolysis in the system up to 100 hours, and that the effects of autolysis become appreciable thereafter.⁶

Returning to the relation between φ and t observed at 25°C. and shown in Fig. 2, observations⁷ made during the first 24 hours show that the curve is a really composite one such as that of Fig. 3, *i.e.* that it begins as a sigmoid curve which approaches an asymptotic value of φ much greater than zero (Fig. 2, inset D), the effects of autoly-

but in these systems the lysin is present in relatively high concentration from the time $t = 0$. In the case of the autolytic systems now under consideration, there is no lysin present at $t = 0$, the lysin appearing and increasing as the time becomes greater. It is therefore very likely that P as well as a is a function of time.

⁶ The change in the course of the curve may also be due to the accidental introduction of bacteria. The relation between φ and t in contaminated systems is often of the type shown in Fig. 2 rather than of the type shown in Fig. 1, even at 4°C.

⁷ Flame photometer determinations have to be carried out very carefully when the initial portions of these curves are being investigated, for the K losses are small during the first few hours. The analysis of the initial portion of the curve at 25°C. (Fig. 2, inset D) shows that the curve starts off as an exponential approaching the asymptote $\varphi_{\infty} = 0.85$ with a constant value of a of 0.057. This gives $P = 0.048$, a higher value than that usually found at 4°C., and so the temperature coefficient of both P , the measure of the activity of the accumulation process, and a , the permeability constant, is positive.

The loss of K during the first hour or two is less than one would expect from the subsequent course of the curve, the relation between φ and t being sigmoid instead of uniformly convex to the t -axis (*cf.* Ponder, 1947 *b*, footnote 4). This is presumably the result of the change in P or in a which causes the system to proceed to a new steady state not being instantaneous, but taking place over the course of an hour or two. It is this portion of the curve which is most likely to suffer distortion, particularly at the higher temperatures, as a result of the liberation of K from rapidly disintegrating white cells and platelets (*cf.* Sheppard and Martin, 1948). Washing the cells reduces the number of white cells to about 50 per cent, and the number of platelets to about 10 per cent, of their initial values, and so this source of error is not as important as it would be in experiments with whole blood.

sis becoming apparent only after this initial period. This is a state of affairs similar to that described by Davson (1937), whose curves, at 40°C., seem to flatten off to asymptotes during the 8 hours to which his observations were limited.

There is some justification, indeed, for regarding the intermediate type of curve, such as that shown in Fig. 3, as being the most general expression of the relation between φ and t . Looked at from this point of view, the curve shown in Fig. 1 constitutes one extreme case in which no lysis is present in the system at $t = 0$ and in which no autolysin ever appears, whereas the exponential curves with $a = \text{constant}$ and $\varphi_{\infty} = 0$ (Ponder, 1948 *b*) constitute the other extreme case, in which a lysis is present in the system from the time $t = 0$, and in which the effects of the lysis are substantially instantaneous.

Demonstration of Presence of Autolysins.—The presence of autolytic substances or of substances which increase autolysis can be shown by replacing the supernatant fluid of a system of human red cells in saline at 4°C. with some of the supernatant fluid from a system of human red cells in saline which has stood for 48 hours at 25°C. The rate of K loss in the first system, which is kept at 4°C. both before and after the substitution of the supernatant fluid from the second system, is compared with the rate of K loss in a system, also kept at 4°C. throughout, the supernatant fluid of which has been left undisturbed.

When the supernatant fluid is left undisturbed, the curve relating φ and t resembles that shown in Fig. 1; *i.e.*, φ is a simple exponential function of t with an asymptote in the neighborhood of 0.5. After a long time, such as 144 hours, φ is accordingly found to be about 0.5. When the supernatant fluid of the system is removed at the end of 48 hours and replaced with an equal volume of supernatant fluid from another system which has been kept at 25°C. for 48 hours and in which lysis has begun,⁸ the curve relating φ and t turns downwards like that shown in Fig. 2, so that at the end of a long time such as 144 hours it has a value in the neighborhood of 0.1 instead of in the neighborhood of 0.5. This shows that autolysins or substances which favor autolysis develop in the system kept at 25°C., and that these substances are transferrable to the system kept at 4°C., in which autolysis does not usually occur in the times under consideration.⁹

⁸ The supernatant fluid contains the K lost at the end of 48 hours by the cells of the system kept at 25°C., and the addition of this K (usually $F = 0.2$ to 0.3) to the system at 4°C. has to be allowed for in calculating the subsequent course of the curve for the system at 4°C.

⁹ The possible nature of autolysins such as these has been discussed elsewhere (Ponder, 1948 *c*), the two most interesting possibilities being that they are substances related to lysolecithin, produced by enzymatic action on the red cell lipids themselves, and that they are substances of the fatty acid class resulting from tissue autolysis. Following a suggestion made by Bianchi (1946), I have added 1 in 2000 quinine to the systems in an attempt to poison the lysolecithin-producing system; greater rather than smaller K losses occurred at all temperatures, probably because 1 in 2000 quinine is a lysis in a hypolytic concentration. NaCN, however, slightly decreases both K loss and spontaneous lysis; it is possible that it does so by inhibiting an enzyme system.

3. *K Losses at 37°C.*

A typical curve for the loss of K from human red cells into 172 m.eq./liter NaCl at 37°C. is shown in Fig. 4, plotted from data (Table IV) treated in the same way as those from which the curves of Figs. 1, 2, and 3 are constructed. So far as the observations made between 2 and 24 hours are concerned, the

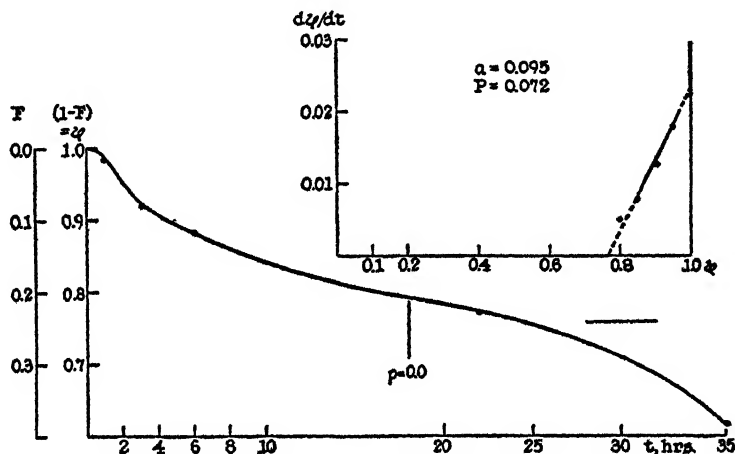


FIG. 4. Loss of K from washed human red cells into 172 m. eq./litre NaCl at 37°C. Ordinates and abscissa as in other figures. Inset, analysis in terms of P and a .

TABLE IV
Relation of K Loss to Time at 37°C.

t	K	ϕ	F
hrs.			
1	0.02	0.00	0.02
3	0.08	0.00	0.08
6	0.12	0.00	0.12
22	0.22	0.03	0.23
36	0.38	0.13	0.29

curve relating ϕ and t is an exponential approaching the asymptote $\phi = 0.76$; it therefore resembles the curve at 4°C. shown in Fig. 1 and the initial portion of the curve at 25°C. shown in Fig. 2, inset D. If observations are continued for more than about 24 hours, the curve begins to bend downwards to become a curve of the "intermediate" type just described. Analysis of the part between $t = 2$ and $t = 24$ gives $\phi_{\infty} = 0.76$, $a = 0.095$, and $P = 0.072$. These values of a and of P are higher than those usually observed at 25°C., which again are higher than those usually observed at 4°C.

The initial portion of the curve is sigmoid. Between $t = 0$ and $t = 2$, it is

concave to the t -axis; after $t = 2$, it becomes convex, and so the relation between φ and t is of the type which would be expected if initial values of P_0 or of a_0 were to change, not quite instantaneously, to the new values which determine the position of the new asymptote $\varphi_\infty = 0.76$. If there were enough experimental points, this region of the curve could be analyzed by the same method as that used in the case of the curve in Fig. 2 or of the curve for the NaF system shown in Fig. 5.

TABLE V
Values of φ_∞ , a , and P at 37°C.

Donor	φ_∞	a	P
1	0.86	0.125	0.108
2	0.82	0.116	0.095
3	0.76	0.095	0.072
4	0.76	0.097	0.070
5	0.69	0.072	0.049
6	0.55	0.055	0.028
7	0.68	0.082	0.056
8	0.65	0.079	0.051
9	0.70	0.100	0.070
10	0.66	0.091	0.061
Average.....			0.066

Table V gives values of φ_∞ and of a found in a series of observations at 37°C. on systems containing the red cells of different donors.

4. Systems Containing Substances Which Affect the Rate of Respiration, Metabolism, Etc.

Given a cation-permeable red cell which maintains a difference between the K content of its interior and the K content of the medium surrounding it by means of a metabolic process, it will be clear from the foregoing that a loss of K will occur if the constant a increases or if the rate of accumulation P decreases. The systems in which resorcinol, guaiacol, or n butyl alcohol are present at $t = 0$ (Ponder, 1948 *b*) are probably instances of cases in which the final result is brought about by an increase in the "permeability constant" a .¹⁰ The

¹⁰ If the curves for the three concentrations of resorcinol, 0.016 M, 0.032 M, and 0.048 M, all reach the asymptote $K = 1.0$ or $\varphi_\infty = 0.0$ in a simple exponential manner, P/a must approach zero in all cases. This may be the result of a decrease of P to zero or of a large increase in a , P remaining relatively constant; the point can be settled only by making simultaneous determinations of metabolic activity. Again, however, some doubt must arise as to whether the curve for the system containing 0.016 M resorcinol, for example, does not approach an asymptote a little higher than $\varphi_\infty = 0.0$, in which case the value of P/a for the system would be greater than zero and P might have a real value; see also the section of "immobile K," below.

analysis does not distinguish between the many possible combinations of variation in a and P , but some further insight into the course of events may be obtained by adding substances, such as fluoride, cyanide, iodoacetate, etc., which are known to affect red cell metabolism.

The effects of these substances will be described as they occur at the two temperatures 4° and 37°C ., since curves at intermediate temperatures are difficult to interpret for the reasons already set forth (section 2, above).

1. *Fluoride*.—Fig. 5 shows the effect on the K loss at 4°C . of replacing half the NaCl of the system with 0.172 M NaF. The curve for the NaCl system approaches the asymptote $\varphi_\infty = 0.54$ exponentially with $a = 0.019$, $P = 0.010$,

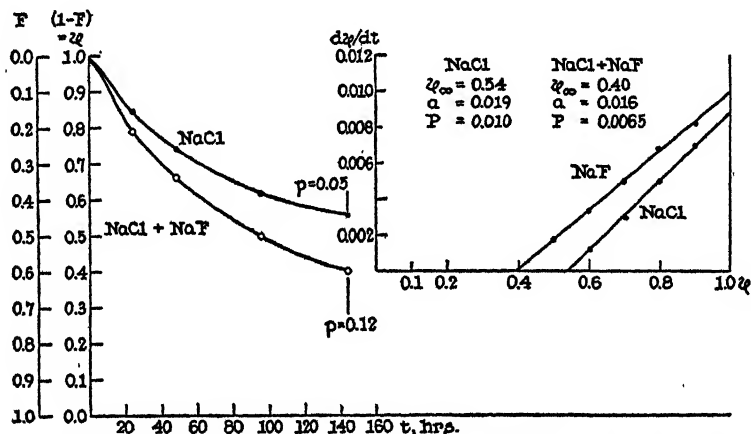


FIG. 5. Effect of 0.086 M NaF on the K loss from washed human red cells into 172 m. eq./litre NaCl at 4°C . Ordinates and abscissa as in other figures. The amount of lysis in the systems at the end of 144 hours is given by the two values of p . Inset, analysis of the curves in terms of P and a .

while the curve for the NaCl + NaF system approaches $\varphi_\infty = 0.40$ with $a = 0.016$, $P = 0.0065$. This result is compatible with the rate of accumulation P being reduced by the presence of fluoride to about 65 per cent of its value in the NaCl system.

Fig. 6 shows the effect on the K loss at 37°C . of replacing half the NaCl of the system with 0.172 M fluoride. The increase in the rate of K loss in the NaCl + NaF system is now very conspicuous, the curve relating φ and t approaching the asymptote $\varphi_\infty = 0.005$ (equilibrium between K inside the cell and K outside) and φ being as small as 0.2 even after 12 hours. Analyzing this curve in the same way as the curve in Fig. 2 is analyzed, a series of values of a corresponding to various values of φ can be found (Fig. 6, inset A, curve marked a). The value of the constant rises from its initial value of 0.02 to a new value of 0.2 as φ falls from 1.0 to 0.7, and the curve marked a in Fig. 6, inset B, shows how the value of a varies with time. Within the first 5 hours it increases from

0.02 to 0.2, a change which would correspond to a relatively slow effect of NaF in increasing the value of a .

This, however, is not the only or even the most likely possibility, for NaF may produce its effect by reducing metabolic activity and the rate of accumulation P . Suppose that a remains constant and that P varies explicitly with time; then, from (1),

$$P = a\varphi - d\varphi/dt \quad (3)$$

i.e., P can be evaluated by drawing tangents to the experimental curve and subtracting their slopes from $a\varphi$. To decide on the value of a which ought to be

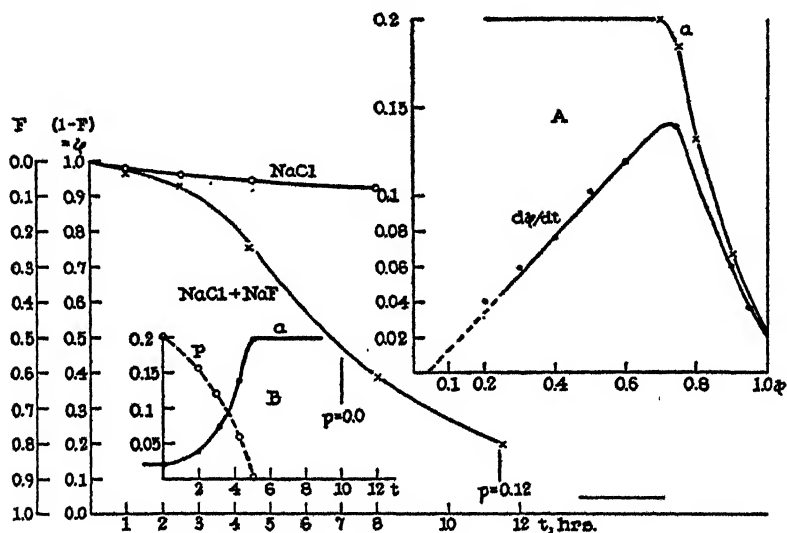


FIG. 6. Effect of 0.086 M NaF on the K loss from washed human red cells into 172 m. eq./litre NaCl at 37°C. Ordinates and abscissa as in other figures. Inset A, values of a , P being assumed to be zero. Inset B, variation of a with time, and of P with time, a being assumed to remain constant.

assumed, let us suppose that $P = 0$ for the lower part of the curve, *i.e.* that the effect of NaF is to reduce the rate of accumulation to zero after it has been acting for 5 hours or more; the constant value of a is then $(d\varphi/dt/\varphi) = 0.2$. The curve starts at $\varphi = 1.0$, which is equal to P_0/a ; the initial value of P_0 at $t = 0$ is accordingly 0.2. From this value it decreases to zero during the first 5 hours of the experiment (Fig. 6, inset B, curved marked P and drawn through circles), the decrease corresponding to a relatively slow effect of NaF in decreasing the rate of accumulation P .

An intelligible enough result is arrived at by looking at the situation in either of these two ways, and there is the further possibility that both a and P , and

not only one of them, may vary explicitly with time. This treatment of the experimental data is therefore more of an inquiry into the possibilities than an analysis, and the next step is obviously to obtain an independent measure of the metabolic activity upon which P is supposed to depend. It would be a great advance if one could establish an understandable relation between the rate of metabolism (perhaps of glycolysis alone) and the events described by a curve such as that marked P in Fig. 6, inset B, because only by doing so is there any likelihood that the interlocked effect of variations in a and in P can be separated from each other.

The effect of fluoride on the K loss increases with the NaF concentration, at first rapidly and then more slowly. When human red cells are used, there is no evidence that the NaF effect goes through the maximum at 0.07 M observed by Davson (1941) in systems containing rabbit red cells.

"Immobile K".—It should be emphasized that in systems containing NaF at 4°C. φ tends towards an asymptote appreciably higher than $\varphi = 0.05$, at which the K content of the cells would be equal to that of the external medium. Some 20 to 40 per cent of the cell K seems to have a diffusibility much smaller than that of the rest of the cell K. A similar conclusion can be arrived at by considering the curves for K loss into NaCl or NaCl-buffer, where the asymptotic value of φ usually lies between 0.4 and 0.6. Translated into values of P , these values require that the cells accumulate about 0.6 per cent of their initial K per hour, *i.e.* about 0.6 m.eq./liter per hour; this is about 10 per cent of the accumulation which would be expected to occur from the glycolytic mechanism at 37°C. working with 100 per cent efficiency, and so is too large to be probable for a system which has stood at 4°C. for 100 hours or more, and which contains no added glucose. Even when glycolysis has been greatly reduced by the addition of NaF, the value of φ may still be between 0.2 and 0.4; it therefore seems likely that part of the cell K is held inside the cell by forces which are not dependent on metabolism (*cf.* Mazia, 1940). It is an attractive idea that these forces are related to the orderly arrangement of materials in the cell surface and interior, for the unexpectedly high position of the asymptote which suggests that some of the cell K is "immobile" is not observed when the systems contain lysins (Ponder, 1948 *b*; see also footnote 8) or in systems containing NaF at 37°C. It is only observations at low temperatures which suggest that a fraction of the cell K is "immobile;" if it should prove to be, the variables to be considered in the description of the curves for K loss as a function of time will be not only the accumulation rate P and the diffusion constant a , but also a quantity (φ_{∞} in the absence of metabolism) which defines how much "immobile K" the cell contains and which may vary with temperature and other factors affecting cell structure.¹¹

¹¹ Another way of accounting for the observations is to suppose that, after a long time (*e.g.*, 100 hours), the value of a becomes much smaller than it was previously. This is essentially Davson's idea that the membrane of the cell goes through a phase of permeability to K and then becomes impermeable. It does not seem likely in view of the increasing hemolysis observed as the cells are allowed to stand for longer

2. *Iodoacetate*.—The effect of sodium iodoacetate in concentrations between 0.02 per cent and 1.0 per cent on the course of K loss is small for the first 5 to 10 hours at 37°C.; after that time the K loss becomes rapid and is nearly complete at the end of 24 hours. As Wilbrandt (1937) has already observed, the effect is delayed as compared with the NaF effect; after 5 hours at 37°C., for example, the value of ϕ may be 0.8 in a system containing 0.02 per cent iodoacetate, while it would have fallen to the neighborhood of 0.3 in a system containing 0.5 per cent NaF.¹²

At 4°C., concentrations of iodoacetate between 0.02 per cent and 1.0 per cent produce an increase in the rate of K loss, the curve usually obtained being one which approaches $\phi = 0.05$ as an asymptote. Only occasionally is the asymptote situated at a higher value (as it regularly is in systems containing NaF). Iodoacetate in these concentrations, however, is quite lytic, e.g. $p = 0.2$ after 144 hours in a system containing 0.02 iodoacetate, and so it is almost certain that the results are complicated by a modification of the cell structure.

3. *Cyanide*.—The addition to the system of M/1000 NaCN (buffered at pH 7.5) does not produce any increase in the rate of K loss either at 4°C. or at 37°C. The rate of loss, indeed, tends to be smaller in systems containing NaCN because of a slight reduction in the amount of spontaneous hemolysis.

4. *Sugars*.—Addition of sufficient glucose to produce a 200 to 400 mg./100 ml. concentration in the system has very little effect on the course of K loss at 4°C. At 37°C., K is taken up by red cells from media into which it has been lost at lower temperatures, provided that glucose is added and that a glycolytic mechanism is active (Harris, 1941; Maisels, 1948). The kinetics of this uptake remain to be studied.

In the systems with which this paper is concerned, i.e. in systems in which 0.8 ml. of red cells loses about 30 per cent of its K into 11.2 ml. of isotonic NaCl during 96 hours at 4°C., the rate of uptake of K by the cells at 37°C. in the presence of added glucose (200 mg./100 ml.) is just about great enough to balance the loss which occurs

and longer times. A possibility which has to be considered more seriously is that fluoride produces irreversible injury to the cells, and that what appears to be an effect on an accumulating mechanism and a reduction in P is really the result of this injury and an increase in the constant a . Still another way of accounting for the observations is to suppose that there are some 30 per cent of red cells which are altogether impermeable to K at low temperatures; i.e., that the K-Na exchanges observed take place in a part of the population only (cf. Ponder, 1947 a). Up to now, no experimental method has been found for excluding this as a possibility.

¹² From 5 to 10 per cent of the washed human red cells in systems containing Na iodoacetate become resistant to hypotonic hemolysis, remaining intact as spheres in a tonicity of 0.2 at 25°C. To prepare standards representing 100 per cent hemolysis, a small amount of K-free lysin, such as Na tetradecylsulfate, should be added.

into glucose-free NaCl at 37°C. Only rarely is it greater; these systems are accordingly unsuitable for the direct measurement of K accumulation, although they are excellent for the measurement of the rate of K loss. Since the rate of K loss at 37°C. is about 1 per cent per hour, the rate of uptake must be of the same order. Maisels (1948) gives uptake rates of about 1.5 per cent per hour for systems containing unwashed red cells and much more K in the supernatant fluids. It will be noticed that these values are considerably less than the 7 per cent per hour which corresponds to the average value of P at 37°C. (Table V). The latter, however, is a value calculated in relation to a supposedly steady state, whereas the former are average values for a process which is probably continually slowing down; the experimental values of 1.0 to 1.5 per cent per hour are accordingly likely to be minimal values.

5. *Hypotonicity*.—Davson (1937) observed that the red cells of all species examined (rabbit, ox, horse, guinea pig, pig, man) lose K when placed in hypotonic NaCl at 40°C.; at lower temperatures, the losses were smaller. The rate of loss (per cent of initial cell K lost in 120 minutes) was found to increase with decrease in tonicity, and the curve relating K loss to time was found to tend towards an asymptotic value far removed from that at which there would be an equilibrium between the K inside the cell and that outside; *i.e.*, to resemble the initial part of the curve shown in Fig. 4 of this paper. Davson made the suggestion that the cell membrane undergoes a transitory increase in permeability as the cell swells to a certain critical volume at which the loss of K becomes rapid; this critical volume would be less than the critical volume for hemolysis, and would be related in some way to the stretching of the cell surface. With the loss of K, the cell would shrink away from this critical volume, the surface would no longer be stretched, and the initial impermeability of the membrane to K would be restored; in this way the loss of a certain fraction of the cell K, and no more, might be explained.¹³ Davson's observations extended over relatively short times (2 to 8 hours); they were made before the extent to which K is lost into isotonic media was appreciated, and the losses into hypotonic media were not systematically compared with those occurring into isotonic media.¹⁴

¹³ The evidence which Davson gives in favor of this explanation is indirect, and involves the assumption that Na does not enter the cell as K leaves it. Analysis of the K and Na contents of the swollen cells, after they have stood at 25°C. in a medium of tonicity 0.6 for 48 hours, during which time about 20 per cent of the cell K is lost, shows that a K-Na exchange has taken place with the final concentration of K + Na equal to about 0.6 times the initial concentration. Davson also considered the possibility that the new steady state reached in the systems might be the result of a metabolic process, but rejected this mechanism on grounds which would not now be thought adequate.

¹⁴ Over and above the K losses under consideration, losses as large as 9 per cent of the initial cell K may occur when red cells are centrifuged (Davson and Danielli, 1938).

In protracted experiments with sterile systems at 4°C., the rate of loss of K from human red cells into hypotonic NaCl is usually less than the rate of loss into isotonic media. At the same time, the value of φ_{∞} is greater, *e.g.* $\varphi_{\infty} = 0.60$, $\alpha = 0.011$ for $T = 1.0$, but $\varphi_{\infty} = 0.65$, $\alpha = 0.009$ for $T = 0.7$. At 37°C. there is no significant difference between the K loss into isotonic NaCl and into hypotonic NaCl of a tonicity $T = 0.6$ or 0.7 , a typical result being $\varphi_{\infty} = 0.76$, $\alpha = 0.097$ in each case.

SUMMARY

Curves describing the loss of K from human red cells as a function of time can be interpreted in terms of an equation which treats the K content of the cell (φ) as the result of an accumulation process occurring at a rate P and an outward diffusion process regulated by a constant α . The equation is useful for describing the observations and for exploring the mechanisms which may be responsible for the K losses, although it cannot be used for analyzing the experimental data in a strict sense in the absence of independent metabolic data because P and α may both be functions of time. The applicability of the equation is illustrated by its use in connection with experimental curves showing K loss as a function of time at 4°, 25°, and 37°C. for systems containing human red cells in isotonic NaCl or NaCl-buffer.

At 4°C., the K loss follows an exponential curve approaching an asymptote in the neighborhood of $\varphi = 0.50 \pm 0.15$. The corresponding value of P implies that the cells are able to accumulate about 0.6 per cent of their initial K per hour under these conditions.

At 25°C., the K loss starts exponentially but becomes roughly linear with time after 24 to 48 hours. The change of form is probably due to the appearance of autolysins in the system. Curves of a similar mixed or intermediate form may be obtained even at 4°C. if the observations are sufficiently extended and if spontaneous hemolysis becomes appreciable.

At 37°C., the K loss is exponential for the first 24 to 36 hours, the curves approaching asymptotes which, translated into terms of P , indicate that the cells can accumulate about 7 ± 3 per cent of their initial K per hour. After this time autolysis begins to affect the shape of the curves, the rate of K loss increasing rapidly.

When the suspension medium is made hypotonic by the addition of water, between 2 and 5 per cent of the initial K is lost within 10 minutes at 25°C. (Ponder and Robinson, 1934), only about 0.5 per cent being lost during the same time into isotonic NaCl. These losses would occur, in these experiments, before the time denoted by $t = 0$. It will be noticed that the initial K concentration K_0 is determined in the cells of a sample of the completed system, and is almost always less than the K concentration in the unwashed red cells of the heparinized blood.

The effect of adding fluoride or iodoacetate is to lower the position of the asymptote to which the curves proceed; *i.e.*, to decrease the accumulation rate P , to increase the diffusion constant a , or both. Cyanide has almost no effect. Hypotonicity has little effect on the rate of K loss at 37°C.; at 4°C., the rate of loss is somewhat less in hypotonic NaCl.

The observation that the K loss in systems at 4°C. and containing as much as 0.086 M NaF does not become complete, but proceeds exponentially towards an asymptote between $\phi = 0.2$ and 0.4, suggests that 20 to 40 per cent of the cell K is much less diffusible than the remainder at low temperatures and in the absence of lytic substances. A similar conclusion is suggested by the form of the curve for K loss into saline at 4°C., an accumulation rate of 0.6 m. eq./litre of cells/hour at the end of 100 hours or more being improbably great for a system at such a low temperature and containing no added glucose.

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PHAGE FORMATION IN STAPHYLOCOCCUS MUSCAE CULTURES

V. FURTHER OBSERVATIONS ON THE RELATIONSHIP BETWEEN VIRUS RELEASE AND CELLULAR LYSIS

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It has previously been reported from this laboratory that in synthetic medium of Fildes containing hydrolyzed casein *Staphylococcus muscae* virus is released without visible lysis of the host cell (1). Lysis of the cell did eventually occur in this system, but only after the phage was released. These observations were confirmed not only by turbidimetric readings but also by direct microscopic examination of the infected cells. It will be shown in this paper that (a) in a synthetic medium with a high multiple infection of virus, virus release is correlated with cellular lysis in contrast to a very low multiple infection in which virus release occurs without visible lysis although the final yield of virus is the same in both cases and (b) a non-dialyzable substance extracted from yeast when added to a cell infected with a very low multiple infection results in virus liberation being correlated with cellular lysis, although the final yield of virus liberated per cell is not affected.

EXPERIMENTAL RESULTS

Fig. 1. shows the effect of a low and high multiple infection on the release of the virus in synthetic medium of Fildes containing hydrolyzed casein. With a high multiple infection, virus liberation is correlated with cellular lysis. With a very low multiple infection virus liberation occurs before observable cellular lysis. The final yield of virus per cell is the same in both instances. Virus liberation begins at the same time in both instances.

The next question was whether the virus particle itself was accelerating lysis in multiple infection or whether something else in the lysate was the active lysing agent. In order to answer this question phage solutions were inactivated in three ways: (a) Shaken at 37°C. at pH 5.0, (b) shaken at 37°C. at pH 9.0, and (c) heated at 60°C. Samples were removed at short intervals. When the phage titer had dropped so that the addition of the phage solution would result in a low multiple infection, the treated solutions were added to bacterial cultures. In all three cases, the virus was liberated before cellular lysis, while in the control tube containing a sample of untreated virus which gave a multiple infection of about 7, cellular lysis occurred 30 minutes sooner and was cor-

related with virus liberation. By centrifugation at 10,000 R.P.M. for 90 minutes, it was also possible to show that the virus particle itself was the active lysing agent. Finally phage, purified by differential centrifugation as described previously (2), also caused the cells to lyse sooner when used in a high multiple infection. All the evidence therefore indicates that it is the virus particle itself which is accelerating cellular lysis under the conditions of a high multiple infection.

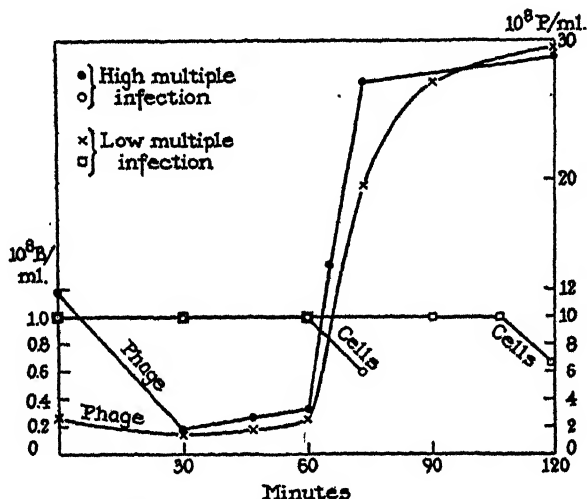


FIG. 1. The effect of high and low multiple infection on the release of a bacterial virus. Two tubes, A and B, containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein were inoculated with 1.0×10^8 cells prepared as described under Methods. They were incubated 1 hour at which time the cell count was 1.1×10^8 cells per ml. Tube A then was inoculated with virus to give 2.8×10^8 virus particles per ml. and tube B was inoculated with virus to give 1.2×10^8 phage particles per ml. Turbidimetric measurements and phage counts were then taken at intervals.

The next experiments were designed to test the theory that lysis observed in a very low multiple infection was due to the readsorption of the released particles which would then result in a high multiple infection and consequently cellular lysis. To do this experiment two tubes, A and B, were set up, with the cells being infected with a very low multiple infection. After 70 minutes, at which time all the virus had been released into the medium, the cells in tube B were centrifuged out and resuspended in fresh synthetic medium. If lysis under conditions of a very low multiple infection was due to the readsorption of released particles, then tube A should have lysed before tube B. However, both tubes lysed at the same time, cellular lysis in tube A beginning at 98 minutes and in tube B, 100 minutes. This experiment was repeated three more times with

tubes A and B lysing not more than 8 minutes apart. These experiments, together with the experiment shown in Fig. 1, in which the virus count continually rises and then reaches a plateau and remains constant, indicate that after cells have released their phage they are no longer capable of readsorbing phage. If the cells were able to readsorb phage, one would expect a drop in the virus titer sometime after the virus was released as is observed when one multiple infects normal cells (*cf.* Fig. 1, tube initially infected with a high multiple infection). This drop in titer did not occur (*cf.* Fig. 1, tube initially infected

TABLE I

The Effect of the Yeast Fraction on Virus Liberation

Two tubes, A and B, were set up containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein. Both tubes were inoculated with 1.0×10^8 cells per ml. Tube A received 0.2 ml. of H_2O and tube B, 0.2 ml. of yeast fraction (340 γ of N). The tubes were then incubated for 1 hour at which time the count was 1.4×10^8 cells per ml. Each tube then received 0.1 ml. of virus solution to give a virus titer of 3.8×10^8 particles per ml. Turbidimetric measurements and phage measurements were then taken every 10 minutes.

Experiment	Sample	γ N of yeast fraction per 10.0 ml.	Multiplicity of infection	Onset of lysis	Beginning of virus liberation	Virus particles liberated per cell
				<i>min.</i>	<i>min.</i>	
1	A	—	1.3	90-100	50-60	31
	B	340	1.6	50-60	50-60	34
2	A	—	1.6	80-90	40-50	38
	B	340	1.1	40-50	40-50	31
3	A	—	1.1	100-110	50-60	41
	B	340	1.7	50-60	50-60	36

with a low multiple infection). However, more work should be done to make certain of this point.

The Effect of the Yeast Fraction

A non-dialyzable fraction was isolated from dry yeast which when added to cells infected with a very low multiple infection resulted in virus liberation being correlated with cellular lysis (Table I). It can also be seen that the yeast fraction accelerates the lysis of the infected cells. There is no effect on the final yield of virus per cell. This indicates that the yeast fraction is concerned with the lytic process and not with virus formation. The yeast fraction has no observable effect on the cells in the absence of the virus.

By varying the concentration of the yeast fraction under the condition of a low multiple infection, it is possible to vary the time of lysis as shown in Table II. It should be noted, however, that no matter at what time the cells begin

TABLE II

The Effect of Varying Concentrations of the Yeast Fraction on Cellular Lysis

The conditions were the same as described in Table I. Varying amounts of the yeast fraction were added to separate tubes. The multiplicity of infection was approximately 1.4 in all samples.

Sample	γ N yeast fraction per 10.0 ml.	Onset of lysis	Beginning of virus liberation	Virus particles liberated per cell
		<i>min.</i>	<i>min.</i>	
1	—	100-110	60-70	40
2	340	60-70	60-70	32
3	170	60-70	60-70	36
4	85	80-90	60-70	43
5	42.5	100-110	60-70	37

TABLE III

The Effect of High Multiple Infection and the Yeast Fraction on the Liberation of the Virus

Four tubes, A, B, C, and D, were set up containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein. All tubes were inoculated with bacteria to give 1.0×10^8 cells per ml. Tube A received 0.2 ml. of water, tube B, 0.2 ml. of yeast fraction containing 340 γ of N of the yeast fraction, tube C, 0.2 ml. of water, and tube D, 0.2 ml. of yeast fraction (340 γ of N). The four tubes were then incubated $1\frac{1}{2}$ hours. At this time the cell count was approximately 1.2×10^8 cells per ml. in all tubes. Tubes A and B were inoculated with virus to give 3.2×10^8 particles per ml. Tubes C and D were inoculated with the virus to give 1.8×10^9 particles per ml. Turbidimetric readings and phage determinations were then carried out every 10 minutes.

Experiment	Sample	γ N of yeast fraction per 10.0 ml.	Multiplicity of infection	Onset of lysis	Beginning of virus liberation	Virus particles liberated per cell
				<i>min.</i>	<i>min.</i>	
1	A	—	1.3	100-110	60-70	41
	B	340	1.8	60-70	60-70	36
	C	—	7.1	60-70	60-70	34
	D	340	6.3	60-70	60-70	31
2	A	—	1.7	110-120	60-70	37
	B	340	1.1	60-70	60-70	31
	C	—	6.3	60-70	60-70	44
	D	340	7.6	60-70	60-70	34
3	A	—	1.2	90-100	40-50	51
	B	340	1.8	40-50	40-50	41
	C	—	6.1	40-50	40-50	46
	D	340	7.2	40-50	40-50	48

to lyse, the yield of virus formed per cell is the same. Furthermore, in all instances, virus release begins at the same time.

Table III shows that infected cells treated with the yeast fraction behave as cells infected with a high concentration of virus. It should be noted that lysis starts approximately at the same time in the latter two instances. The addition of the yeast fraction to cells infected with a high number of virus particles does not cause lysis to occur sooner than without the yeast fraction.

In Table IV the effect of adding the yeast fraction at various times to cells with a very low multiple infection is illustrated. The sample to which the

TABLE IV

The Effect of Adding the Yeast Fraction at Various Times on Virus Liberation from the Host Cell

Three tubes, A, B, and C, were set up containing 10.0 ml. of synthetic medium plus 5.0 mg. of hydrolyzed casein. The three tubes were then inoculated with 1.0×10^8 cells per ml. Tube A then received 0.2 ml. of water and tube B, 0.2 ml. of yeast fraction (340 γ of N). The tubes were then incubated $1\frac{1}{2}$ hours at which time the cell count was 1.2×10^8 cells per ml. All three tubes were then inoculated with virus to give 3.4×10^8 particles per ml. Phage counts and turbidimetric readings were then taken every 10 minutes. After 45 minutes, tube C received 0.2 ml. of the yeast fraction (340 γ of N).

Experiment	Sample	γ N yeast fraction per 10.0 ml.	Beginning of lysis	Beginning of virus liberation	Virus particles liberated per cell
			<i>min.</i>	<i>min.</i>	
1	A	—	110–120	50–60	41
	B	340 at beginning	50–60	50–60	34
	C	340 after 45 min.	90–100	50–60	30
2	A	—	100–110	40–50	51
	B	340 at beginning	40–50	40–50	49
	C	340 after 45 min.	80–90	40–50	53

virus and yeast factor had been added at the same time began to lyse in 50 minutes. The sample to which the yeast fraction was added 45 minutes after the virus began to lyse in 90 to 100 minutes. The tube without yeast factor began to lyse in 110 to 120 minutes. This experiment shows that the yeast factor does not have to be added at the same time as the virus to influence the lytic reaction.

Experiments were also carried out to determine whether the yeast fraction could influence cellular lysis after phage formation had stopped. Three tubes, A, B, and C, were inoculated with cells containing a very low multiple infection (about 1.3). Tube B also received the yeast fraction. At 60 to 70 minutes, when tube B began to lyse and presumably virus formation had stopped, tube C received the yeast fraction. Tubes A and C both began to lyse at 100 to 110 minutes. Experiments of this type are difficult to interpret since the time at

which the control tube A began to lyse is about when one would have expected tube C to lyse if the yeast factor could influence the lytic process after virus formation had stopped. The question whether the yeast fraction can influence cellular lysis after virus formation has stopped must remain open.

DISCUSSION

The experiments reported in this paper support the view that cellular lysis in the *S. muscae* system in synthetic medium of Fildes containing hydrolyzed casein may or may not accompany virus liberation depending upon the conditions employed in the experiment.¹ With a very low multiple infection the virus is released before the cells begin to lyse. With a high multiple infection or with the addition of a yeast fraction to cells infected with a low multiple infection, virus liberation is directly correlated with cellular lysis and occurs much sooner than the lysis observed in cells infected with a low multiple infection. The results indicate that in a high multiple infection it is the virus particle itself that accelerates the lytic process when compared to the lytic process observed in a very low multiple infection. One interpretation of this result could be that the virus contains a lytic enzyme, and thus the cells infected with a larger number of particles would lyse sooner than a cell infected with a single particle. Further speculation about the other points raised by these experiments does not appear advantageous at present.

The rôle of the yeast fraction in cellular lysis is obscure at this time. All that can be said is that it accelerates the lytic process under the condition of a very low multiple infection and does not affect the number of virus particles formed. In such a system the effect of the yeast fraction on the lytic process is similar to the effect produced by the addition of more phage particles. By adding different concentrations of the yeast fraction, it is possible to lyse the cells at varying times. No matter when the cells lyse under these conditions, the yield of virus per cell is the same within experimental error. The yeast fraction has no observable effect on the cells in the absence of the virus.

Under the above three conditions, *i.e.* cells infected with a low multiple infection, cells infected with a low multiple infection plus yeast factor, and cells infected with a high multiple infection, although the lysis time greatly varies, the final yield of virus per cell is the same within experimental error. This result is further evidence for the theory that cellular lysis is an accessory phenomenon in virus formation rather than a process directly correlated with the increase in number of virus particles formed in the cell. Evidence for such a

¹ This view also receives support by the following observation. A low multiple infection of cells in the log phase, instead of in the physiological state used in the experiments described in this paper, results in cellular lysis occurring at an earlier interval and being correlated with virus liberation.

theory has also been obtained by Fowler and Cohen (3) working with the *E. coli* system. These investigators found that the addition of certain amino acids would influence cellular lysis with little or no effect on the formation of the virus.

The fact that the virus yield per cell is the same in cells which have such varying times of lysis indicates that virus formation proceeds up to a certain point and then stops before the cells begin to lyse. This observation may mean that there is some substrate in the cell, the exhaustion of which causes virus formation to stop. The recent experiments on *E. coli* by Luria and Latarjet (4) using ultraviolet irradiation and by Latarjet (5) using x-rays could also be taken as evidence that there is some substrate in the cell, the depletion of which results in the cessation of virus reproduction.

Methods

The same *S. muscae* system was used as described previously (1). Bacteria and virus were grown and determined as described earlier (2). All calculations of virus particles formed per cell under the condition of a multiple infection are based on the phage counts after complete lysis had taken place according to the method of Delbrück and Luria (6).

The beginning of lysis as shown in Tables I to IV was determined by taking the time the first drop in turbidity was noted in the Klett-Summerson colorimeter. Since all the cells were infected in these experiments, there was no cellular multiplication and the turbidity remained constant until lysis occurred. The time of lysis determined by the turbidimetric method corresponded to the time of cellular lysis observed under the microscope.

Preparation of the Yeast Fraction.—30 gm. of Fleischmann's pure dry yeast type 20-40 was suspended in 120 ml. of distilled water and brought to 70°C. The pH was then adjusted to 5.5 with glacial acetic acid. The yeast was extracted 1 hour at 70°C. and then filtered with suction. The filtrate was dialyzed against a large volume of distilled water for 12 hours at 5°C. Further purification of the active substance could be achieved by acid precipitation at pH 4.0, with the active compound being precipitated.

This work was carried out with the technical assistance of Mr. M. Litovchick and Mr. E. Wenzlaff.

SUMMARY

1. In the synthetic medium of Fildes containing hydrolyzed casein virus release is not correlated with observable cellular lysis under conditions of a very low multiple infection.

2. In cells with a high multiple infection, lysis occurs much sooner than in cells with a low multiple infection and virus release is correlated with cellular lysis. The experiments indicate that it is the virus particle itself which accelerates lysis under these conditions.

3. A non-dialyzable fraction has been isolated from yeast, the addition of which results in cellular lysis occurring at a sooner than usual interval and being correlated with virus release in cells having a very low multiple infection.

4. By varying the concentration of the yeast fraction, it is possible to lyse the cells at varying times under conditions of a low multiple infection.

THE STATE OF THE CHROMOSOMES IN THE INTERPHASE NUCLEUS

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PLATES 1 AND 2

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The present knowledge of chromosomes is derived mainly from a study of cells in mitosis, where they are well defined individual structures. Much less is known about the chromosomes in the non-dividing nucleus where they appear to lose their characteristic structure and individuality. In the living resting nucleus there is usually nothing visible except nucleoli. After fixation, however, a great variety of images are produced—finely or coarsely granular structures or a network of fibers with smaller or larger clumps of chromatin. Even in the same nucleus the structure can vary greatly according to pretreatment and mode of fixation. Therefore very little has been learned about interphase chromosomes from a study of fixed preparations. Since individual chromosomes cannot be recognized in the resting nucleus even the evidence that they persist from one mitosis to the next is mainly indirect. It is based on the fact that chromosomes usually do not change their position in the resting nucleus so that they reappear in prophase in the same place as they were seen at preceding telophase (5, 9).

Recently we have isolated chromosomes from resting nuclei of mammalian tissues (33). They are bodies of characteristic size and shape, visibly double, with tightly spiraled and more or less unspiraled regions. This is a direct confirmation of the view that chromosomes persist as individual structures in the interphase nucleus and this method makes it possible to study cytologically chromosomes from resting nuclei. It is at first surprising that chromosomes from interphase nuclei look so much like mitotic chromosomes, since it is usually assumed that chromosomes unravel and become completely dispersed at telophase. In mammalian tissues like thymus, liver, pancreas, kidney, this is, however, not the case. Only certain parts of the mitotic chromosomes are unraveled in the resting nucleus, others remain tightly spiraled. These thick and darkly staining regions correspond to the heterochromatic lumps seen in fixed nuclei. Structures similar to isolated chromosomes can be recognized in fixed nuclei, or nuclei isolated in citric acid, but since so many chromosomes are packed together in a nucleus they are all tangled up and cannot be seen as individual structures. The comparison of isolated chromosomes with isolated citric acid nuclei (or otherwise fixed nuclei) shows that they have not been changed essentially by the techniques of isolation, except perhaps by fragmenta-

tion. Such fragmentation cannot be common, since morphologically characteristic types of chromosomes are found repeatedly.

In the living uninjured nucleus, however, no structures can be seen as has been pointed out by many investigators of living cells. What, then, are the changes which the chromosomes undergo during isolation in physiological saline or during fixation? What is their structure in the living interphase nucleus? We find in the literature two main points of view. One places emphasis on the apparent lack of structure in the living nucleus and holds that the living nucleus does not contain formed chromosomes but two colloidal substances, chromatin and karyolymph, which are evenly dispersed. Upon fixation a separation of the two phases takes place resulting in the familiar fixation images. We may call this the *colloid hypothesis* (16, 19, 37, 40, 45). The other viewpoint emphasizes the genetic continuity of the chromosomes and holds that chromosomes exist in the living nucleus very much as they are seen in fixed preparations. They are not visible because, it is supposed, chromosomes and surrounding karyolymph have the same refractive index (21, 30, 34, 41). The best evidence for this *hypothesis of structural equivalence* of the living and the fixed nucleus comes from photographs of living cells taken with ultraviolet light of wave length 2600 Å, the absorption maximum of nucleic acid (14, 29, 43, 44). These photographs seem to show that in living cells the distribution of chromatin (*i.e.*, desoxyribonucleic acid = DNA) is the same as in fixed cells. Such photographs are, however, pertinent only if it is certain that the cells were alive and uninjured at the time when the photograph was taken. The best evidence that a cell is alive is a continued normal mitosis. Another criterion is the appearance of the nucleus in visible light, for it is well known that uninjured nuclei are usually without visible structure but that upon injury structural elements appear. Such a change in the appearance of nuclei is caused by ultraviolet radiation (4, 7). Therefore, it is necessary to demonstrate that during the exposure to ultraviolet light the appearance of the nucleus in visible light has not changed. The ultraviolet photographs of "living cells" published so far lack such a proof that the cells were not injured during preparation and exposure to the ultraviolet light. It is apparent then that the ultraviolet photographs hitherto published have given no decisive evidence on the structure of the living interphase nucleus. This has also been pointed out recently by Brumberg and Larionow (11). Using undoubtedly living cells they reached entirely different conclusions than had previous workers. Their results will be discussed later in this paper.

1. *Ultraviolet Photographs of Living Cells.*—In studying the chromosomes in living nuclei with ultraviolet photographs it is important to choose cells which can be demonstrated clearly to be alive and which, furthermore, have large chromosomes rich in desoxyribonucleic acid, since the absorption of chromosomes at 2600 Å depends mainly on the presence of this substance. Spermato-

cytes of grasshoppers have large chromosomes and can be followed through normal divisions in suitable preparations.

Testis follicles of a grasshopper (*Melanoplus femur-rubrum*) were teased in a drop of Belar's solution¹ (6) and the cells mounted in a hanging drop preparation. Suitable cells were then photographed at 2537 Å using a G. E. germicidal lamp (4 watt) with quartz condensing lens and a Bäckström filter² (1).

In such a preparation one finds among the prophase cells (pachytene) two types of different appearance. In some cells the chromosomes are highly refractile definite structures as in fixed cells, in others, on the other hand, no definite chromosomes are visible. In Fig. 2 are shown three cells from the same cyst and therefore in the same stage of prophase. In two of them the chromosomes are distinct, in the third cell only hazy shadows can be seen. A photograph at 2537 Å gives essentially the same picture, two cells with clearly visible, absorbing chromosomes, one with diffuse absorption throughout the nucleus (Fig. 1). These three cells were then irradiated with ultraviolet light (2537 Å) for 20 minutes and photographed again in visible light (Fig. 4) and at 2537 Å (Fig. 3). We see that as a result of the irradiation the nucleus which absorbed diffusely now shows distinct chromosomes both in the visible and the ultraviolet light. From a comparison of these figures it is evident that photographs at 2537 Å show definite chromosomes only when they are already distinct in visible light. Nuclei appearing homogeneous in visible light show diffuse absorption in the ultraviolet. Distinct chromosomes can be seen only in cells which are clearly injured, either mechanically during teasing or through ultraviolet irradiation. Such cells do not continue to divide. In pachytene cells which are alive and continue mitosis the nuclei appear homogeneous and absorb diffusely at 2537 Å.

Another material often used for the study of living cells is the epidermis of onion bulb scales. The epidermis can easily be stripped free and mounted on a slide in a drop of tap water. In freshly prepared epidermis only nucleoli, but no chromosomes are visible. A photograph at 2537 Å shows the diffuse absorption of the nucleus (Fig. 5). If the preparation has been standing for a while or if dilute acetic acid is added to the water, a change takes place in the nucleus. A mass of thin coiled threads becomes visible. Upon fixation with 45 per cent acetic acid the nucleus shrinks and the chromosome threads become thicker and more highly refractile. A photograph at 2537 Å gives the same picture, the absorption being localized in definite chromosome structures (Fig. 6).

Ultraviolet photography of grasshopper spermatocytes prophases and inter-

¹ NaCl (9 per cent) 20 cc.; KCl (1 per cent) 4 cc.; CaCl₂ (1 per cent) 4 cc.; NaHCO₃ (10 per cent) 0.4 cc. Distilled water up to 200 cc.

² The filter used with the germicidal lamp consists of a quartz cell 5 cm. in length filled with an aqueous solution of 28 per cent NiSO₄ and 8 per cent CoSO₄.

phase nuclei of onion epidermis thus gives entirely different pictures when the cells are alive than when they are injured or dead. Furthermore, ultraviolet photographs do not reveal chromosomal structures in living cells where none are seen in visible light. The difference in appearance of the nucleus when alive or dead is therefore not primarily due to a change in refractive index, but to a change in the distribution of the material with a high absorption at 2537 Å. This is the desoxyribonucleic acid (DNA) of the chromosomes. When it is distributed evenly through the nucleus as in the living cell we shall refer to such a nucleus as being in the *extended state*. When the DNA is localized in typical chromosomal structures as in fixed cells it will be referred to as the *condensed state* of the nucleus.

2. *The Effect of Electrolytes and Non-Electrolytes on the Structure of the Interphase Nucleus.*—That the extended state of the interphase (and also prophase) nucleus is extremely labile has been demonstrated by a number of authors since the reversible appearance and disappearance of chromatin structures was described by Lewis (27) and van Herwerden (23). Almost any interference with the cell causes the appearance of visible structures in the nucleus. In order to study the state of the chromosomes in the living nucleus and the nature of the changes upon injury, conditions must be found which will make the state of the living nucleus more stable. Cohen (17) described experiments in which nuclei of onion scale epidermis were dissected out in various media. He found that in sucrose, glycerin, and distilled water nuclei looked structureless as in the uninjured cell. Bancher (2) reported the same for glucose. On addition of salts or acid Cohen saw the chromatin structures appear as in fixed nuclei.

In order to determine whether the structure of nuclei teased in sucrose corresponds to that in living cells, grasshopper spermatocytes and onion epidermis cells were teased in 10 per cent sucrose and photographed at 2537 Å. Figs. 7 and 8 compared with Figs. 1 and 5 demonstrate the identity in appearance of living nuclei and dead nuclei in sucrose solution. If such nuclei are teased out in physiological salt solutions they have the same structure as after fixation.

If some methyl green is added to the sucrose solution the nuclei stain diffusely green. Upon the addition of acetic acid the chromosomes appear and the green stain is now limited to these structures. Since it has been shown that methyl green stains specifically the DNA of the nucleus, this is further evidence that the DNA is evenly distributed in the extended state and localized in the visible chromosomes in the condensed state of the nucleus.

Next we isolated nuclei from mammalian tissues by teasing a small piece of a drop of 10 per cent sucrose on a slide. Fig. 9 shows a rat liver nucleus photographed at 2537 Å. In sucrose the nucleus looks perfectly homogeneous. If now a drop of 0.8 per cent NaCl is added to the slide the chromatin structure appears and the nucleus shrinks (Fig. 10). If the salt is washed out with sucrose the nucleus becomes homogeneous again (Fig. 11). This process can be

repeated several times. Figs. 12 and 13 show the appearance of calf thymus nuclei in sucrose and after fixation. Figs. 14 and 15 represent beef liver nuclei in sucrose and after fixation.

The nuclei of salivary glands in Diptera are of special interest because of their peculiar banded appearance and their significance in cytogenetic work. They are rather unusual as interphase nuclei since the chromosomes are clearly visible as individual structures after fixation. If glands are dissected out in 10 per cent sucrose only the nucleolus is visible in the nucleus. By gently pressing on the cover glass the nuclei can be squeezed out of the cell. Even so they retain their homogeneous appearance. If methyl green is added to the sucrose the nuclei stain evenly green, only the nucleolar area being unstained (Fig. 23).

These observations then lead to the conclusion that the extended state of the nucleus is not peculiar to the living cell. Even in dead nuclei the chromatin can exist either in an extended state or condensed into chromosomal structures depending on the medium. In non-electrolytes (glucose, sucrose, glycerin) the chromatin is extended, in electrolyte solution it condenses.

3. The Effect of Electrolytes and Non-Electrolytes on Isolated Chromosomes.—What is the nature of this reversible change in the distribution of DNA in the nucleus? Does the DNA go on and off the chromosomes or do the chromosomes themselves swell and contract?

To answer this question chromosomes from calf thymus resting nuclei were isolated in 0.8 per cent NaCl and suspended in sucrose. Even in the test tube the difference in appearance of the chromosomes in salt and sucrose is striking. The suspension of chromosomes in sucrose is much less opaque than in saline and greater centrifugal force is necessary to spin them down. Under the microscope individual chromosomes are almost invisible. If some methyl green is added they stain green and one can see now that they have the same general shapes but are greatly swollen (Fig. 17). Upon addition of 0.8 per cent NaCl they shrink and look again as they did originally (Fig. 16). As with nuclei the change is reversible. This change in volume of the chromosomes can be measured directly. Equal amounts of isolated chromosomes were suspended in 0.8 per cent NaCl and in 30 per cent sucrose. They were then centrifuged down until no further change in volume of the chromosome mass took place. In sucrose the volume of the precipitate is four to five times greater than in saline. The swelling and shrinking which were observed on nuclei in sucrose and salt solutions (Figs. 9–11) is therefore the result of the volume changes in the chromosomes. When the chromosomes condense the nucleus shrinks and the nuclear membrane often looks shrivelled (Fig. 10). If the chromosomes are made to extend again they fill the nucleus and expand it so that the nuclear membrane becomes tight and smooth. Analysis of chromosomes in saline and after standing in sucrose overnight shows that no DNA comes off the chromosomes when

they swell (Table I). Some pentose phosphorus is lost, caused by slight autolysis of the residual chromosome (33).

The behavior of the chromosomes in sucrose was then compared with that of residual chromosomes (*cf.* reference 33), that is to say chromosomes from which the histone and practically all the DNA have been removed. These residual chromosomes look the same whether they are suspended in sucrose or saline. This is further evidence that the DNA is responsible for the change in the state of the chromosomes.

These experiments on isolated chromosomes demonstrate clearly that the change observed in the appearance of nuclei is actually a change in the state of the individual chromosomes themselves. The highly polymerized DNA which makes up a large part of most chromosomes forms a gel-like structure which can reversibly extend and condense.

4. *The Effect of Salt Concentration on Interphase Chromosomes.*—If interphase chromosomes isolated from mammalian tissues are suspended in 1 M NaCl a

TABLE I
Total Nucleic Acid P and Pentose P of Two Preparations of Calf Thymus Chromosomes in Saline and after Being Suspended in 30 Per Cent Sucrose Overnight

Total nucleic acid P in per cent dry weight		Pentose P in per cent total nucleic acid P	
Saline	Sucrose	Saline	Sucrose
3.75	4.04	2.2	0.57
4.05	4.21		

thick gel is formed. Microscopic study reveals that this gel is due to the swelling of the individual chromosomes. Upon rapid stirring the gel breaks and the DNA and histone go into solution. Nuclei of onion scale epidermis behave similarly. If teased out in 1 M NaCl the nuclei become homogeneous, they swell until the membrane breaks, and the nucleolus floats out into the cytoplasm. Sometimes the nucleus swells considerably before bursting. The nuclear contents then separate into two fractions, a cap-like mass of threads and a clear area (*Kappenplasmolyse*, *cf.* references 3, 40). The fibrous mass consists most likely of the residual chromosomes, while in the clear vacuole the DNA and histone are in solution.

If nuclei are fixed in acetone followed by 95 per cent alcohol the DNA condenses, but in 1 M NaCl the nucleus becomes again homogeneous, staining diffusely with methyl green. After several hours in 1 M NaCl in the cold the nuclei no longer stain with methyl green, which indicates that the DNA has gone into solution.

The effect of salts on nuclei and chromosomes then depends on the concentration of the salts. In very low concentrations the chromosomes are in the ex-

tended state, in physiological concentration they condense. In higher concentration they extend again until finally the DNA goes into solution. The behavior of nuclei and chromosomes thus parallels the behavior of isolated nucleohistone (32).

5. *Fixation of Nuclei in the Extended State.*—After treatment with most common fixatives the interphase nuclei show definite structure; the chromatin is fixed in the condensed state. But it has been observed many times that the structure of interphase nuclei varies with different fixatives. After fixation with formalin and osmic acid for instance the nuclei were often found to be quite homogeneous. Formalin and osmic acid thus preserve the extended state of the uninjured living nucleus. To prevent occasional condensation after formalin fixation we found the following method most useful. Before fixation it must be made certain that the nuclei are in the extended state, either in uninjured cells or, with dead material, in sucrose. The material is then fixed in 20 per cent formalin (1 part neutral formalin plus 4 parts water). Time of fixation depends on the size of the material. The formalin is replaced with 0.2M lanthanum acetate for several hours. After careful washing with water the nuclei can be stained with Feulgen.

Fig. 18 shows a nucleus of onion scale epidermis fixed with formalin and stained with Feulgen. The nucleus stains diffusely and only the nucleoli are unstained. The cytoplasm and the nucleoli are perfectly clear. Fig. 19 represents a nucleus which was in the condensed state before fixation, treated in the same fashion. The chromosome threads are clearly visible. In Fig. 21 we see a pachytene of the grasshopper fixed in the extended state and stained with Feulgen. The Feulgen picture is identical with the ultraviolet photograph of a living nucleus (Fig. 1). The substance absorbing diffusely at 2537 Å in a living nucleus is therefore DNA. Isolated chromosomes in sucrose can be fixed in the extended state by adding formalin to the sucrose suspension.

6. *Distribution of Protein in the Extended State.*—After the nuclei are fixed in the extended state a cytochemical test for proteins can be performed (35). Only the distribution of total protein can be shown, since after fixatives which preserve the extended state the histone can no longer be removed with acids.

Fig. 20 shows a nucleus of the onion scale epidermis, fixed in the extended state, treated with trichloroacetic acid-Millon reagent and photographed at 3650 Å. A marked general absorption of the nucleus and the absence of structure are apparent. Therefore, in the extended state we find not only the DNA but also protein, diffusely distributed through the nucleus.

7. *The Behavior of Chromosomes with Low DNA Concentration.*—If the reversible extension and condensation of chromosomes are due to their DNA content, then we should expect that chromosomes with a very low concentration of DNA would behave differently from the chromosomes discussed so far. The lampbrush chromosomes in the oocyte of the frog contain so little DNA that

they hardly stain with Feulgen or methyl green. Germinal vesicles of frog eggs were dissected out in 10 per cent sucrose. The chromosomes are faintly visible as delicate threads forming the characteristic loops. If the nuclei are dissected out in sucrose to which a little pyronin is added, the chromosomes begin to stain red and stand out most clearly (Fig. 24). Since pyronin in high concentration precipitates nucleic acid, the solution used was tested on a piece of calf liver. The liver nuclei, teased in this solution, remained completely extended. The pyronin, therefore, did not cause the appearance of chromosome threads. Germinal vesicles dissected out in sucrose were also fixed with 20 per cent formalin. The lampbrush chromosomes were as clearly and distinctly visible as after fixation in Carnoy (alcohol-acetic acid). Furthermore, the fixation did not distort the appearance of the chromosomes, they looked no different from those in sucrose.

Chromosomes poor in DNA thus do not show reversible condensation and their structure is not visibly altered through handling or fixation. They behave in the same way as residual chromosomes prepared from isolated calf thymus chromosomes.

We can now summarize the evidence that the DNA is responsible for the reversible extension and condensation of chromosomes. In living nuclei we have found a diffuse absorption throughout the nucleus at 2537 Å. Nuclei isolated in sucrose stain evenly with methyl green. After fixation with formalin or osmic acid the nuclei stain diffusely with Feulgen. Thus in the extended state the DNA is distributed throughout the nucleus. Finally residual chromosomes and lampbrush chromosomes which contain very little DNA do not show the reversible condensation.

8. *The Nuclei of Chick Fibroblasts in Tissue Culture.*—In 1946 Brumberg and Larionow (11) published some very interesting ultraviolet photographs of living and dead cells in tissue culture. Using a reflecting objective they did not have to expose the cells to the ultraviolet except in taking the photograph. They found that the nuclei of living cells absorb very little at 2600 Å. Only after the cells were killed by longer exposure to the ultraviolet did nuclear structures become visible which appeared to absorb at 2600 Å. They concluded that the DNA either did not absorb in the living nucleus or that it was differently distributed.

We have repeated and extended these observations on cultures of chick embryo fibroblasts.³ In order not to expose the cells unnecessarily to the ultraviolet the focussing was done in visible light and then adjusted for 2537 Å by moving the fine adjustment a definite number of units which had been determined empirically. We found that after taking a photograph at 2537 Å the cells went through normal mitosis and were therefore alive at the time of exposure. A group of cells were then killed by prolonged exposure to the ultraviolet and photographed again. The pictures of living cells were found to be

³ These cultures were kindly prepared for us on quartz slides by Dr. Ruth Hoffman.

strikingly different from those of cells killed with ultraviolet. In living fibroblasts the cytoplasm appears dark, the nucleus much lighter and without structure except for nucleoli. In the dead cells, however, the cytoplasm is very light, but the nucleus now stands out clearly with definite membrane and chromosomal structure. So far then our observations agree with those of the Russian workers. But it must now be determined whether the darker appearance of the dead nucleus is due to specific absorption at 2537 Å as Brumberg and Larionow assumed or caused by an increase in structural light loss due to a change in refractive indexes. Nucleic acids can be removed in cytological preparations by heating at 90°C. in 0.3 M trichloroacetic acid for 15 minutes (35). A culture of chick fibroblasts was therefore fixed in acetic-alcohol and a group of cells photographed at 2537 Å. The nucleic acids were then removed in hot trichloroacetic acid and the same cells photographed again at 2537 Å. After the trichloroacetic acid treatment the nucleoli and the cytoplasm were markedly less dark, but the appearance of the chromatin had not changed. It follows that some of the dark appearance of the cytoplasm and nucleoli was due to nucleic acid. The appearance of chromatin structures in the dead nucleus, however, is not caused by specific absorption, but by structural light loss due to a change in refractive indexes. This means that the nucleus of these cells contains little DNA, while the cytoplasm is rich in ribonucleic acid. Therefore, the nucleus is lighter than the cytoplasm in ultraviolet photographs of living cells. When a cell is killed with ultraviolet the ribonucleic acid leaks out of the disintegrating cytoplasm and the refractive index of nuclear membrane and chromatin increases over that of the surrounding medium. Therefore, in ultraviolet photographs the nucleus appears now darker than the cytoplasm and with definite internal structure. Brumberg and Larionow were certainly correct in their observations that ultraviolet photographs of living and dead cells are strikingly different. But the cells they chose contain so little DNA that they are unsuited for the study of the nature of any changes in the distribution of nucleic acids. Nuclei with a higher concentration of DNA had to be used for that.

9. *The State of Chromosomes during Mitosis.*—During nuclear division, when the chromosomes are moved about in the cell, they occupy only a small volume of the nucleus. Since they fill the entire nucleus during interphase, they must decrease in volume in addition to the spiralization which occurs during prophase. Such a decrease in volume can be effected easily through a condensation of the DNA during mitosis. In living cells the chromosomes are usually visible during metaphase and anaphase. The change in appearance during fixation is small compared with that of the resting nucleus. This indicates that indeed a condensation of the DNA has taken place. But it is only partial, since a further condensation can still occur in mitotic chromosomes with weak acids, or through asphyxiation, without killing the cell. In fresh hanging drop preparations of grasshopper spermatocytes the chromosomes are faintly visible.

After some time the chromosomes become more refractile and sharply outlined. The same effect is observed if the pH of the medium is lowered with CO_2 or dilute acetic acid (pH 5-6). Such cells can still finish mitosis normally.

Fig. 22 shows a metaphase of the first spermatocyte division fixed with formalin-lanthanum acetate and stained with Feulgen. It comes from the same preparation as Fig. 21 which shows a pachytene nucleus in the extended condition. The metaphase chromosomes appear somewhat swollen and with hazy outlines compared with similar chromosomes fixed in acid fixatives.

In prophase chromosomes therefore a partial condensation of the DNA takes place together with the coiling of the chromonemata. During telophase this is reversed again into the maximally extended state of the interphase nucleus.

DISCUSSION

The discoverer of the nucleus, Robert Brown, described it as a clear vesicle within every cell. Since then many investigators studying living cells in both animals and plants found the nucleus to be without visible structure except for the nucleoli. The use of fixatives and dyes allowed a detailed analysis of the morphology and the complicated behavior of the chromosomes during cell division. But only conflicting results were obtained with regard to the structure of the resting nucleus, especially since little was known about the changes which take place in it during fixation. Engelmann (20) and Flemming (21) already knew that even unfixed nuclei could look quite differently depending on the medium. The reversible appearance and disappearance of chromosome structures in the living nucleus were first clearly described by Lewis (27) and van Herwerden (23) and since then a large number of papers have been published describing the various conditions under which structure appears in previously homogeneous nuclei. It was thus established that structures seen in fixed preparations are invisible in uninjured nuclei. This situation has been explained mainly in two ways: (1) The *colloid hypothesis* assumes that the chromatin exists in the resting nucleus in colloidal dispersion and not in individually persisting chromosomes (16, 19, 36, 40, 45). The evidence for this view is as follows: Agents causing the appearance of nuclear structures are also coagulants of nucleoproteins. The nucleus can be fixed in the extended state with osmic acid or formalin and stained with Feulgen. Such preparations show the DNA in these nuclei to be evenly distributed throughout the nucleus (17, 31, 42). In concentrated salt solutions the nuclear content is separated into two phases, karyolymph (sol) and chromatin (gel) (microdissection experiments of Strugger (40) and Bancher (3)). Microdissection shows that most nuclei are filled with a highly viscous substance, but not with chromosomal bodies (4, 26). (2) The *equivalence hypothesis* on the other hand holds that chromosomes exist in the living nucleus as individual structures in a more or less despiraled state and similar to the way they are seen in fixed and stained cells. It is based mainly on the evidence of genetic continuity of chromosomes and observations

on the position of chromosomes in telophase and following prophase (5, 9). Ultraviolet photographs of assumedly living cells showing typical chromatin structures absorbing at 2600 Å were thought to be definite proof for this point of view.

There can hardly be any serious doubt today that chromosomes are persistent structures and that changes in their individuality are rare events caused by spontaneous breakage or under experimental conditions (x-ray, etc.). But the cytological evidence has been indirect only, since it is usually impossible to recognize individual chromosomes in the non-dividing nucleus. With the preparation of morphologically distinct chromosomes from mammalian tissue cells the direct cytological evidence for the individual persistence of chromosomes has been produced. The problem now was no longer whether chromosomes persist during interphase, but in what state they exist in the living nucleus and what changes they undergo upon fixation. Ultraviolet photographs of uninjured cells and the Feulgen staining of formalin-fixed nuclei show that the colloid hypothesis was correct in assuming an even distribution of DNA in the nucleus. But the behavior of isolated chromosomes in electrolyte and non-electrolyte solutions demonstrates that this does not contradict the assumption of individual persistence of the chromosomes. Depending on the state of the highly polymerized DNA each chromosome exists either in an extended or condensed form. Nucleohistone itself behaves in a similar fashion towards electrolytes and non-electrolytes as was pointed out by Jeener (24) who therefore suggested that the properties of nucleohistone might explain the various aspects of nuclei and chromosomes. But the nucleus and the chromosomes are not simply gels of nucleohistone. Chromosomes consist of a complex system of non-histone protein, DNA, and histone with a definite structure. Properties of chromosomes can therefore be studied only on intact chromosomes and not on nucleohistone gels or nucleohistone fibers. Thus, even though the behavior of chromosomes in the living cell can be imitated with nucleohistone in various concentrations of inorganic ions, we know nothing as yet about the conditions in the living nucleus which cause the chromosomes to extend or condense, nor about the possible meaning of the extended state for the functioning of the chromosomes in the metabolic nucleus.

During mitosis, when the chromosomes become tightly coiled, a partial condensation of the DNA takes place. Therefore, chromosomes are generally visible in living dividing cells. Agents which cause condensation in interphase nuclei can cause a further condensation and therefore an increase in refractivity of mitotic chromosomes. This explains the observations of cytologists who found that the visibility of chromosomes changes with pH, tonicity of the medium, mechanical injury, etc. (8, 12, 36). In telophase, together with the despiralization, the chromosomes extend again, so that they all touch each other and can no longer be seen individually. This process has been observed many times in living cells (*cf.* reference 28) and the chromosomes were said to swell

into separate vesicles. In some cases, especially during cleavage, such separate chromosome vesicles or karyomeres are clearly visible during interphase and some authors concluded from this that in every resting nucleus the chromosomes exist as vesicles, even if no membranes can be seen (25, 28). Karyomeres in prophase, however, show clearly that the karyomere membrane behaves like a nuclear membrane and not like part of the chromosome. The chromosome condenses and spirals inside the karyomere membrane which breaks down in later stages of mitosis (*cf.* reference 18). Whether a membrane is formed around a chromosome group or around individual chromosomes probably depends on whether the chromosomes are close together or widely separate at telophase. In any case the chromosomes of the resting nucleus are not vesicles surrounded by a membrane.

The state of the chromosomes in the interphase nucleus naturally determines its viscosity. In most tissue nuclei with a high concentration of DNA the chromosomes fill the entire nucleus. Therefore, nuclei were found to be filled with a highly viscous mass which could be pulled into fibers with the micro-manipulator (4, 26). Egg nuclei, however, were described as containing a liquid of low viscosity (22). These are nuclei with a very low concentration of DNA and where the chromosomes fill only a small part of the nuclear volume.

Stedman and Stedman (38) have recently broached the hypothesis that DNA exists mainly in the nuclear sap and does not form an integral part of the chromosome. Their view was based on an unusual interpretation of the Feulgen reaction. With regard to this they have been answered adequately (10, 13, 15, 39). But it must be pointed out here that the even distribution of the DNA in the living nucleus demonstrated in this paper is entirely different from Stedman's assumption. We have shown that the DNA forms an integral part of the chromosomes and cannot be dislodged from them without breaking chemical bonds, and that the uniform distribution is the result of the swelling of the individual chromosomes, so that they fill the entire volume of the nucleus.

SUMMARY

In the living interphase nucleus no chromosomal structures are visible. Yet in the injured cell and after treatment with most histological fixatives chromatin structures become apparent. Under certain conditions this appearance of structure in the living interphase nucleus is reversible.

We have found that this change in the interphase nucleus is the result of a change in the state of the chromosomes. In the living nucleus the chromosomes are in a greatly extended state, filling the entire nucleus. Upon injury the chromosomes condense and therefore become visible. At the same time the nuclear volume decreases. This behavior of the chromosomes is connected with their content of desoxyribonucleic acid (DNA). This view is based on the following observations:

(a) *Distribution of DNA in the Nucleus.*—(1) The living interphase nucleus

of uninjured cells absorbs diffusely at 2537 Å. No chromosomal structures are visible in ultraviolet photographs unless they are also distinct in ordinary light. If the chromosomes are made to condense they become visible and the absorption at 2537 Å is now localized in these structures. (2) After fixation with formalin and osmic acid interphase nuclei stain diffusely with Feulgen. These fixatives preserve the extended state of the chromosomes. (3) If nuclei are teased out in non-electrolytes (sucrose, glycerin) the chromosomes are extended. Such nuclei stain homogeneously with methyl green. On adding salts the chromosomes condense and the methyl green is now restricted to the visible structures.

(b) *Extension and Condensation of Isolated Chromosomes*.—When chromosomes isolated from interphase nuclei of calf thymus are suspended in sucrose, their volume is four to five times larger than in saline, but they retain their characteristic shapes. Chromosomes from which DNA and histone have been removed do not show this reversible extension and condensation, neither do lampbrush chromosomes of frog oocytes which contain very little DNA.

During mitosis a partial condensation of the DNA occurs in prophase, so that the mitotic chromosomes now occupy a much smaller volume of the nucleus. At telophase the chromosomes swell again to fill the entire nucleus.

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EXPLANATION OF PLATES

PLATE 1

FIGS. 1 and 2. Grasshopper spermatocytes in prophase, photographed at 2537 Å and 436 mμ. One uninjured and two injured cells. Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 × (2537 Å); 600 × (436 mμ).

FIGS. 3 and 4. Same cells, after 20 minutes' irradiation with ultraviolet (2537 Å).

FIGS. 5 and 6. Nucleus of onion epidermis cell, photographed at 2537 Å, living and fixed with acetic acid. Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 ×.

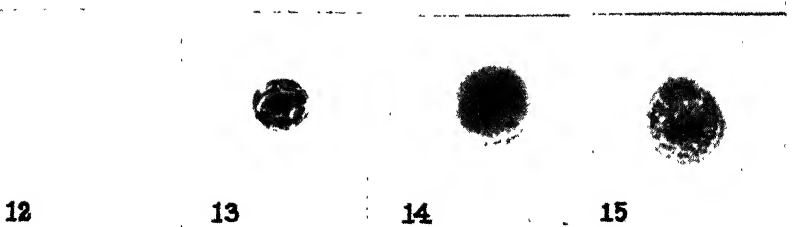
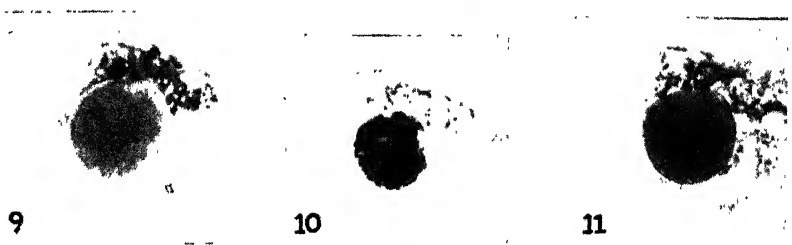
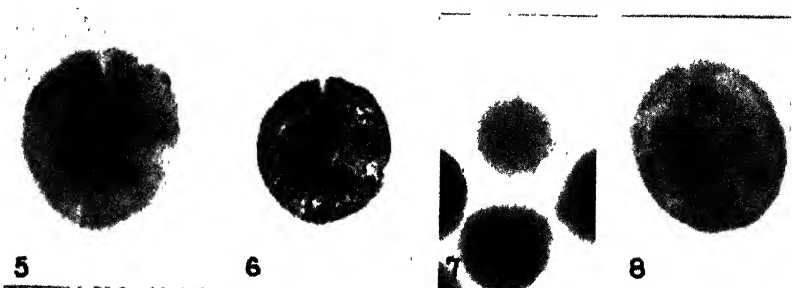
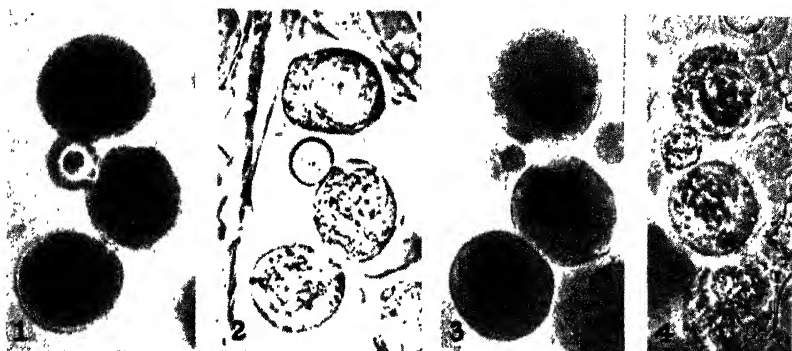
FIG. 7. Grasshopper spermatocyte prophase, killed with ultraviolet radiation (2537 Å) suspended in 10 per cent sucrose. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 ×.

FIG. 8. Onion epidermis nucleus, teased out in 10 per cent sucrose. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 900 ×.

FIGS. 9, 10, and 11. Rat liver nucleus, teased free in 10 per cent sucrose (9); suspended in 0.8 per cent NaCl (10); salt washed out with 10 per cent sucrose (11). Reversible condensation of chromosomes in electrolyte solution. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 1800 ×.

FIGS. 12 and 13. Calf thymus nuclei, suspended in 10 per cent sucrose and after fixation in Carnoy. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 1800 ×.

FIGS. 14 and 15. Beef liver nuclei, suspended in 10 per cent sucrose and after fixation in Carnoy. 2537 Å, Zeiss 2.5 mm. quartz objective, 10 × quartz ocular. 1800 ×.



(Ris and Mirsky: State of chromosomes in interphase nucleus)

PLATE 2

FIGS. 16 and 17. Isolated chromosomes from calf thymus, stained with methyl green in 0.8 per cent NaCl (16) and in 30 per cent sucrose (17). 630 m μ , Zeiss 2 mm. objective, 15 \times ocular. 1200 \times .

FIG. 18. Onion epidermis nucleus, fixed in extended state with formalin and lanthanum acetate, stained with Feulgen. 546 m μ , Zeiss 2 mm. objective 15 \times ocular. 1200 \times .

FIG. 19. Onion epidermis nucleus treated as in Fig. 18, but nucleus was in condensed state before fixation. 546 m μ , Zeiss 2 mm. objective, 15 \times ocular. 1200 \times .

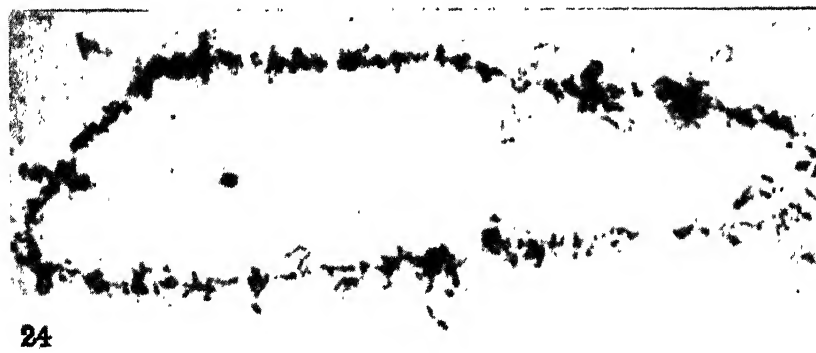
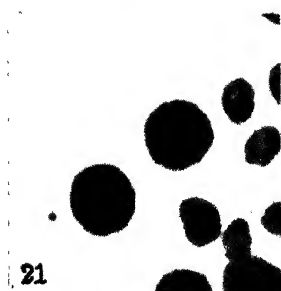
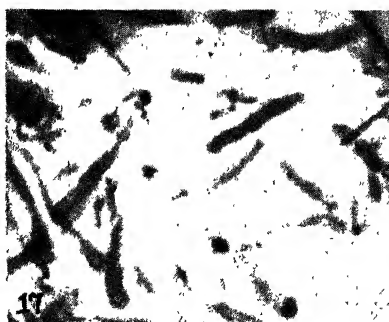
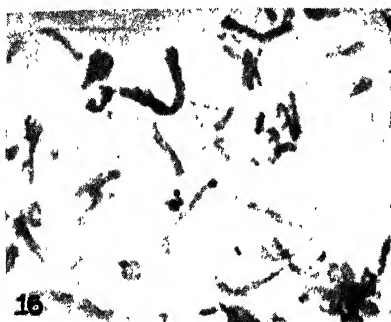
FIG. 20. Onion epidermis nucleus, fixed in extended state with formalin and lanthanum acetate, treated with trichloroacetic-Millon to show distribution of protein. 365 m μ , Zeiss 2 mm. objective, 10 \times ocular. 860 \times .

FIG. 21. Grasshopper spermatocyte prophases, fixed with formalin and lanthanum acetate, stained with Feulgen. 546 m μ , Zeiss 2 mm. objective, 15 \times ocular. 1200 \times .

FIG. 22. Grasshopper spermatocyte, metaphase of first meiotic division. Fixed in formalin and lanthanum acetate, stained with Feulgen. 546 m μ , Zeiss 2 mm. objective, 15 \times ocular. 1200 \times .

FIG. 23. *Drosophila pseudoobscura*, salivary gland nucleus teased out in 10 per cent sucrose, stained with methyl green. 630 m μ , Zeiss 2 mm. objective, 15 \times ocular. 1200 \times .

FIG. 24. Frog oocyte lampbrush chromosome. Germinal vesicle isolated in 10 per cent sucrose, stained with pyronin in sucrose. 546 m μ , Zeiss 2 mm. objective, 15 \times ocular. 2400 \times .



DINITROCRESOL AND PHOSPHATE STIMULATION OF THE OXYGEN CONSUMPTION OF A CELL-FREE OXIDATIVE SYSTEM OBTAINED FROM SEA URCHIN EGGS

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(Received for publication, November 19, 1948)

Various substituted phenols have been found to produce, in low concentrations, a two to three hundred per cent increase in the oxygen consumption of sea urchin eggs. As the concentration of a given substituted phenol is increased, the rate of oxygen consumption passes through an optimum and then declines to or below the normal rate. At concentrations larger than that for optimum oxygen consumption these agents produce a reversible block to the cell division of the fertilized eggs (1-3).

The mechanism of the two phases of the substituted phenol action, namely, the stimulation of oxygen consumption on the one hand and the inhibition of oxygen consumption and reversible block to cell division on the other hand, has received intensive study over the past several years. The analysis of the first phase of the effect has been hampered by the fact that the oxidative stimulation has been obtainable only with intact cells. Efforts to account for the second phase of the effect have revealed that the substituted phenols, when used at adequate concentrations, inhibit the following enzyme systems and cellular activities: *d*-amino acid oxidase (a flavoprotein) (4), *Zwischenferment* and cytochrome reductase (5), growth (6), and phosphate uptake by yeast cells (7), nitrogen assimilation (8), formation of adaptive enzymes (9), and certain other synthetic reactions (10).

Recently, Loomis and Lipmann (11), using a cell-free kidney granule preparation of the type previously employed by Green, Loomis, and Auerbach (12), have reported observations which bear on both phases of the substituted phenol effect. They found that 2,4-dinitrophenol at a concentration of 8×10^{-5} M substantially increased the oxygen consumption of this cell-free preparation. However, when the 2,4-dinitrophenol was used at a higher concentration, 1.8×10^{-4} M, the oxygen consumption was reduced to a normal level and inhibition of aerobic phosphorylation was observed. This observation was of especial interest in its bearing on the mechanism of action of substituted phenols for two reasons: first, it was the first case in which a stimulation of oxygen consumption in a cell-free system had been obtained by the use of substituted phenols; and, second, the inhibition of phosphorylation associated with a decrease in oxygen consumption may provide an explanation for the above mentioned inhibition of oxidative synthetic reactions which are dependent, directly or indirectly, on aerobic phosphorylation (13).

In view of the broad implications of the observations of Loomis and Lipmann, it seemed of interest to see whether either or both phases of the effects on the kidney granule preparation could be repeated with an analogous cell-free oxidative system obtained from the sea urchin egg, where there is the possibility of correlating observations on oxidative synthetic reactions with the mitotic activities of the egg.

The present paper deals with the study of effects of dinitroresol and phosphate on oxygen consumption of a cell-free oxidative system obtained from the eggs of the sea urchin, *Arbacia punctulata*, which was carried out during the summer of 1948.

Experimental Methods

Mature *Arbacia* eggs were obtained at Woods Hole during July and August, 1948. They were shed, washed, and, where necessary, fertilized as described in previous papers from this laboratory (2, 14).

Preparation of Cell-Free Oxidative System from Arbacia Eggs.—While Lipmann and Loomis, in preparing a cell-free oxidative system from mammalian kidney, had at their disposal the information as to how such a cyclophorase system might be obtained (Green, Loomis, and Auerbach, 12), at the time that this work was undertaken no information was available as to how such a system could be obtained from sea urchin eggs. The cyclophorase system obtained from kidney was found to be extremely sensitive to the action of chemical and physical agents. It was destroyed by freezing, drying, or exposure to concentrations of salt greater than 0.1 N or solvents such as alcohol and acetone in concentrations greater than 10 per cent by volume or acidity below pH 4 or alkalinity above pH 10. The procedure employed by Green, Loomis, and Auerbach in obtaining a cyclophorase system from mammalian kidney was not found to be applicable in the case of the *Arbacia* eggs. After various procedures had been tried the following method was finally adopted:—

50 cc. of a 2 per cent suspension of unfertilized eggs in sea water was centrifuged at 1000 g for 2 minutes. The supernatant fluid was poured off and the packed eggs were chilled to about 5°C. in an ice water bath and all subsequent operations prior to the beginning of the manometric observations were carried out with ice cold solutions and containers. The packed eggs were admixed with 3 cc. of a solution containing 0.17 M KCl and 0.23 M sodium citrate (pH 7.7).¹ The resulting dense egg suspension was then alternately drawn into and forced out of a syringe through a No. 18 needle three times; this procedure broke up 95 per cent or more of the eggs.

The suspension of egg fragments was then further diluted with 2 cc. of 0.5 M KCl and centrifuged at 2000 g for 10 minutes in chilled cups. The centrifuging produced a hazy pinkish white supernatant layer, which constituted the cell-free oxidative system and was used as described below, and two layers of sediment which were discarded, a dark red layer at the bottom of the tube² and a yellow yolk layer.

¹ In later experiments the pH of the KCl-citrate was adjusted to 7.0 with no difference in the results.

² This dark red layer contained some egg cells which were not fragmented by the

The method adopted in securing a cell-free oxidative system from fertilized eggs differed slightly from that employed with unfertilized eggs. Fifteen minutes after the eggs had been fertilized, 50 cc. of a 2 per cent suspension of the eggs was centrifuged at 1000 g for 2 minutes. The supernatant liquid was poured off and 3 cc. of a solution containing 0.17 M KCl and 0.23 M sodium citrate was added to the chilled, packed eggs. The egg suspension was allowed to stand for 3 minutes; the fertilized eggs were then broken up in the same manner as previously described for unfertilized eggs, except that the eggs were drawn into and forced from the syringe ten times. The suspension was diluted with 2 cc. 0.5 M KCl, centrifuged as described for unfertilized eggs, and the supernatant layer used as the cell-free oxidative system.

Measurement of Oxygen Consumption by the Cell-Free Oxidative System.—The main compartment of each Warburg flask contained the components shown in Table I, all of which had been held at 4°C. and were admixed in the Warburg vessel, the oxidative system being added last. The center well contained 0.2 cc. 10 per cent KOH.

After the flasks were filled they were attached to the manometers, placed in the bath at 20°C., and shaken at 60 cycles per minute with a 6 cm. amplitude. A 5 minute equilibration period was used prior to the beginning of the measurement.

EXPERIMENTAL RESULTS

Stimulation of Oxygen Consumption by 4,6-Dinitro-o-Cresol and/or Added Inorganic Phosphate.—The rates of oxygen consumption of cell-free oxidative systems obtained from both fertilized and unfertilized *Arbacia* eggs and set up as described in Table I, (A) alone, (B) with addition of dinitrocresol, (C) with addition of phosphate, and (D) with addition of both dinitrocresol and phosphate, are presented in Fig. 1.

With oxalacetate³ as substrate a concentration of 6.4×10^{-5} M 4,6-dinitro-o-cresol (DNC) produced a marked stimulation of oxygen uptake by these cell-free oxidative systems, the stimulation being most marked during the period 30 to 60 minutes after addition of the reagent; with the preparation from unfertilized eggs, no stimulation was evident until 30 minutes after the measurements started. The maximum stimulation induced by the added DNC was approximately 50 per cent in both cases. It is of particular interest that the rate of oxygen uptake by these cell-free oxidative systems from either unfertilized⁴ or syringe treatment and the majority of the egg echinochrome in the form of unbroken pigment granules.

Preliminary experiments, in which the eggs were homogenized without removal of echinochrome, were unsuccessful. Accordingly, the above method of fragmentation of the eggs without destruction of pigment granules was employed. This procedure was first called to our attention by Dr. M. J. Kopac (15).

³ Succinate, α -ketoglutarate, glutamate, and excess citrate were also found to be oxidized by the cell-free oxidative system, but oxalacetate gave the largest increase over the basal level without added substrate.

⁴ In a single exploratory experiment, carried out in duplicate, the oxygen consumption of a cell-free oxidative system prepared from unfertilized eggs previously frozen

fertilized eggs is, in absence of added DNC, about twice that of the intact unfertilized eggs and half that of the intact fertilized eggs.

TABLE I
Contents of Main Compartment of Warburg Flasks

Reagents	Final concentration of reagents* in experiments			
	A	B	C	D
	mM/liter	mM/liter	mM/liter	mM/liter
KCl.....	360	360	360	360
MgCl ₂	14	14	14	14
Glycyl glycine (pH 7.0).....	3.6	3.6	3.6	3.6
Oxalacetate.....	2.9	2.9	2.9	2.9
Glucose.....	36	36	36	36
NaF.....	18	18	18	18
Cytochrome C†.....	0.014	0.014	0.014	0.014
Adenosine triphosphate§.....	0.7	0.7	0.7	0.7
4,6-dinitro- <i>o</i> -cresol (pH 7).....	—	0.064	—	0.064
Sorensen phosphate (pH 7.4).....	—	—	0.7	0.7

These components plus 0.2 cc. of hexokinase|| were admixed and the volume made up to 2.2 cc. with water. Then 0.6 cc. of the cell-free oxidative system was added last.¶

The total Mg⁺⁺ plus Ca⁺⁺ of eggs used approximated the total citrate added.

The cell-free oxidative system in each flask was equivalent to 100 c.mm. eggs or 450,000 to 500,000 eggs. This volume of eggs would consume at 20°C. about 8 c.mm. oxygen per hour in the unfertilized state and about 31 c.mm. per hour in the fertilized state.

* The actual reagents added were: 0.3 cc. 3.34 M KCl, 0.2 cc. 0.2 M MgCl₂, 0.2 cc. 0.05 M glycyl glycine, 0.2 cc. 0.04 M oxalacetate, 0.1 cc. 0.5 M glucose, 0.2 cc. 0.25 M NaF, 0.2 cc. 0.0002 M cytochrome C, 0.2 cc. 0.01 M adenosine triphosphate, 0.2 cc. 0.009 M 4,6-dinitro-*o*-cresol, 0.2 cc. 0.01 M Sorensen phosphate, hexokinase, and oxidative system as shown.

† Cytochrome C was "Injection cytochrome C" purchased from Wyeth Incorporated. This contained 10 mg. per cc. cytochrome C and was diluted on the basis of a molecular weight of 12,000. The preparation also contained 0.01 per cent sodium ethyl mercurithio-salicylate, which gave a final concentration of 4.8×10^{-6} M in each Warburg flask. A control experiment showed that the presence of this mercurial does not account for the absence of phosphorylation by the enzyme system.

§ Adenosine triphosphate was the tetrasodium salt purchased from Rohm and Haas, which is stated by the manufacturer to be 99 per cent pure.

|| The hexokinase used was prepared according to Berger, Slein, Colowick, and Cori (16). The purification was carried through the alumina adsorption stage. The resultant product was diluted approximately 3:1 with water before use.

¶ The cell-free oxidative system contained 0.3 M KCl and 0.14 M citrate. Consequently, the final KCl concentration was raised from the above stated 0.36 M to 0.424 M and citrate was present at a concentration of 0.03 M.

at -20°C. was higher than that of a similar cell-free system obtained from the chilled but unfrozen eggs. The oxygen consumption of the system from the frozen eggs was not increased by addition of dinitroresol or phosphate, but the oxygen consumption of the system from the unfrozen eggs was raised to that of the former by addition of either dinitroresol or phosphate.

Added inorganic phosphate also produced a stimulation of oxygen consumption by both systems and the addition of DNC with phosphate produced only a limited further stimulation in the fertilized system and little or no further stimulation in the unfertilized. These differences may possibly be in part accounted for by the fact that the phosphate originally present in the two systems differed, being higher in the unfertilized than in the fertilized system, with the result

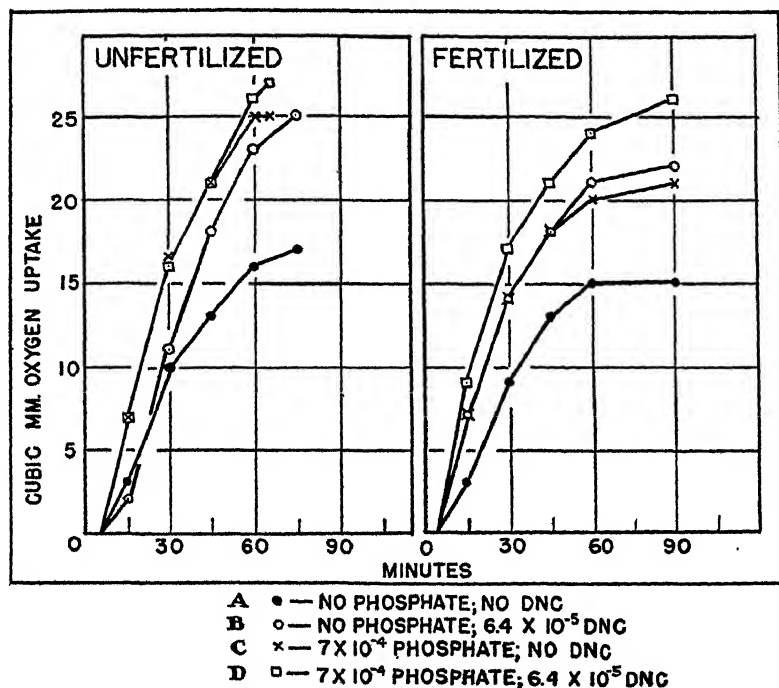


FIG. 1. Effect of 6.4×10^{-5} M 4,6-dinitro-*o*-cresol and/or 7×10^{-4} M inorganic phosphate upon oxygen consumption by cell-free oxidative systems from unfertilized and fertilized *Arbacia* eggs.

that in the fertilized system the phosphate added brought the total content of phosphate to a point between the initial and final levels of phosphate in the unfertilized system.

The stimulation of oxygen consumption by dinitrophenol or phosphate observed by Loomis and Lipman (11) with a cell-free particulate system from rabbit kidney has therefore been confirmed by the present observations with comparable cell-free systems prepared from both unfertilized and fertilized *Arbacia* eggs.

The major portion of the summer season at Woods Hole was expended in determining the conditions under which a cell-free oxidative system capable of

being stimulated by nitrophenols or phosphate could be obtained from *Arbacia* eggs. By the time that a method of obtaining such a system had been worked out the supply of available sea urchin eggs was exhausted and consequently it was necessary to postpone until next season experiments using different concentrations of dinitrophenol to determine whether there was an optimum point of oxygen consumption corresponding with the optimum points for the stimulation of the respiration of sea urchin eggs and of the Lipmann-Loomis oxidative phosphorylating system.

Preliminary Attempts to Obtain a Cell-Free, Aerobic, Phosphorylating System from Arbacia Eggs.—Attempts to determine conditions under which a cell-free, oxidative, phosphorylating system may be obtained from sea urchin eggs have thus far been unsuccessful, but will be resumed when experimental material is once more available. As stated above, no information was available in the literature as to how such a cell-free, phosphorylating system could be obtained from sea urchin or other marine eggs. The establishment of working conditions to produce such a system must necessarily precede the testing of the effect of substituted phenols on the phosphorylating mechanism.

In the experiments illustrated in Fig. 1 the ability of the system to transform inorganic phosphorus was also measured. No net disappearance of inorganic phosphorus was observed and the data are not being reported at present. As shown in Table I, the reaction mixtures used in the experiments presented in Fig. 1 contained glucose as an ultimate phosphate acceptor and yeast hexokinase to transfer phosphate from ATP to glucose. Other experiments were conducted in which succinate, α -ketoglutarate, glutamate, or excess citrate was substituted for oxalacetate as substrate. Also, arginine was introduced as a possible phosphate acceptor, but none of these procedures led to a recognizable phosphorylation.

The authors wish to thank Dr. G. H. A. Clowes, of this laboratory, and Dr. M. E. Krahle, of Washington University, for their advice during the course of this investigation.

SUMMARY

1. A cell-free system capable of using oxygen with oxalacetate as substrate has been prepared from both unfertilized and fertilized sea urchin eggs. The oxygen uptake by this system is about twice that of an equivalent quantity of intact unfertilized eggs and half that of an equivalent quantity of intact fertilized eggs.
2. The oxygen consumption of this cell-free oxidative system can be stimulated by addition of suitable concentrations of 4,6-dinitro-*o*-cresol or by inorganic phosphate. This confirms, with a cell-free system obtained from sea urchin eggs, the observations of Loomis and Lipmann regarding stimulation of oxygen consumption by a system obtained from rabbit kidney.

3. A preliminary but unsuccessful attempt has been made to determine the conditions under which cell-free, aerobic, phosphorylating systems may be obtained from either unfertilized or fertilized sea urchin eggs.

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THE EFFECT OF TEMPERATURE ON POTASSIUM EQUILIBRIA IN CHICK EMBRYO MUSCLE*

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(Received for publication, December 1, 1948)

Cohn and Brues (2) have described a technique employing radioactive isotopes by means of which the rate of exchange of potassium and phosphate ions between the intra- and extracellular phases of tissue cultures could be measured. In the present paper, this technique is employed to study rates of exchange of potassium in chick embryo muscle cultures at different temperatures, and also to study the effect of lowering temperature on the total amount of potassium contained within the cells of cultures.

Methods and Calculations

Minced leg muscle taken from chick embryos of 9 to 11 days' incubation was explanted in a thin plasma clot on the window of a roller bottle, as described by Shaw, Kingsland, and Brues (3). The medium consisted of 4 ml. of modified Baker's peptone medium (4). The explant was allowed to incubate overnight at 37°C. to permit injured cells to repair or disintegrate. At the start of an experiment, the medium was exchanged for one containing radioactive potassium (K^{42}), but otherwise of identical composition. Radioactivity in the culture was measured at 15 to 60 minute intervals by means of a Lauritsen electroscope or Geiger-Muller counter placed opposite the window of the culture bottle. The direct measurements of radioactivity external to the window were used to determine the actual amount of K^{42} in the cultured cells during the course of the experiment, by a method described previously (2). At the end of the experiment, the medium was withdrawn and, when necessary, activity measurements were made and total potassium determined on the medium. When tissue analyses were desired, the tissue was washed twice *in situ* by gentle rocking with 0.85 per cent saline solution for 15 seconds, then removed, ashed with nitric acid, and analyzed for radioactivity and potassium content. Total potassium was determined according to the method of Shohl and Bennett (5) as modified by Hald (6). From these final analyses, the relative specific activity (SA) of the tissue mass at the termination of the experiment was determined, and its specific activity during the course of the experiment was calculated from the readings. Determinations of the rate of radioactive potassium uptake were made at 37°, 26°, 15°, 10°, and 5°.

* An abstract of this paper has been published previously (1).

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In order to study the effect of lowering temperature on total cell potassium, cultures were first allowed to reach an approximate radioactive equilibrium with the medium at 37°. Subsequent losses or gains in activity as the culture is shifted to a lower temperature and returned to 37° represent mass movement of potassium out of or into the cells. Cultures at equilibrium at 37° were lowered to 26°, 15°, 10°, and 5°; readings were continued over a period of hours at the lower temperature and while they were subsequently returned to 37°.

Throughout the experiments, the relative specific activity (SA) of tissue is expressed as a per cent of the specific activity of the entire system of culture plus medium, so that, using cultures of approximately equal mass, all results can be expressed on the same scale. From calculations in the previous paper (2),

$$SA = 100 \cdot S_i / S,$$

where $S_i = K^{42}/K$ in cells, and $S = K^{42}/K$ in the entire system.

Since it was shown previously that within the limits of experimental error (about 10 per cent), all the cell potassium is exchangeable at 37°, a further simplification is possible. Analyses of tissue and medium have shown that, under the conditions employed, this exchange is virtually complete ($SA = 96$) at about 9 hours, and 85 per cent complete ($SA = 85$) at 5 hours. Subsequent uptake curves could then be fitted by setting equal to 85 per cent the level of activity attained at 5 hours at 37°. Similarly, at the end of experiments at low temperatures (15°, 10°, 5°), the cultures were warmed to 37° for several hours in order to establish a reference level of essentially complete exchange.

RESULTS

The loss of potassium from tissue on changing cultures from 37° to 10° or to 5° is illustrated in Fig. 1. Cultures which have reached radioactive equilibrium at 37° show no apparent loss of activity when lowered to 15°, but show a loss of about 12 per cent when lowered to 10° and a 36 per cent loss when lowered to 5°. This loss can be explained only on the basis of a mass shift of potassium from cells to medium at the lower temperatures. The lost activity is rapidly regained on restoring the cultures to 37°, indicating that potassium has re-entered the cells. None of the cultures were followed longer than 9 to 11 hours at low temperatures.

Uptake curves of radioactive potassium by the tissue cultures at 26°, 15°, 10°, and 5° are drawn in Fig. 2. All curves are drawn from pooled data. Because of the method of attainment of equilibrium, that is by warming the cultures to 37° at the end of each experiment, the curves at 10° and 5° are given in terms of the amount of potassium present in the cells at 37° rather than in terms of the

In order to study the effect of lowering temperature on total cell potassium, cultures were first allowed to reach an approximate radioactive equilibrium with the medium at 37°. Subsequent losses or gains in activity as the culture is shifted to a lower temperature and returned to 37° represent mass movement of potassium out of or into the cells. Cultures at equilibrium at 37° were lowered to 26°, 15°, 10°, and 5°; readings were continued over a period of hours at the lower temperature and while they were subsequently returned to 37°.

Throughout the experiments, the relative specific activity (SA) of tissue is expressed as a per cent of the specific activity of the entire system of culture plus medium, so that, using cultures of approximately equal mass, all results can be expressed on the same scale. From calculations in the previous paper (2),

$$SA = 100 \cdot S_i / S,$$

where $S_i = K^a/K$ in cells, and $S = K^a/K$ in the entire system.

Since it was shown previously that within the limits of experimental error (about 10 per cent), all the cell potassium is exchangeable at 37°, a further simplification is possible. Analyses of tissue and medium have shown that, under the conditions employed, this exchange is virtually complete ($SA = 96$) at about 9 hours, and 85 per cent complete ($SA = 85$) at 5 hours. Subsequent uptake curves could then be fitted by setting equal to 85 per cent the level of activity attained at 5 hours at 37°. Similarly, at the end of experiments at low temperatures (15°, 10°, 5°), the cultures were warmed to 37° for several hours in order to establish a reference level of essentially complete exchange.

RESULTS

The loss of potassium from tissue on changing cultures from 37° to 10° or to 5° is illustrated in Fig. 1. Cultures which have reached radioactive equilibrium at 37° show no apparent loss of activity when lowered to 15°, but show a loss of about 12 per cent when lowered to 10° and a 36 per cent loss when lowered to 5°. This loss can be explained only on the basis of a mass shift of potassium from cells to medium at the lower temperatures. The lost activity is rapidly regained on restoring the cultures to 37°, indicating that potassium has re-entered the cells. None of the cultures were followed longer than 9 to 11 hours at low temperatures.

Uptake curves of radioactive potassium by the tissue cultures at 26°, 15°, 10°, and 5° are drawn in Fig. 2. All curves are drawn from pooled data. Because of the method of attainment of equilibrium, that is by warming the cultures to 37° at the end of each experiment, the curves at 10° and 5° are given in terms of the amount of potassium present in the cells at 37° rather than in terms of the

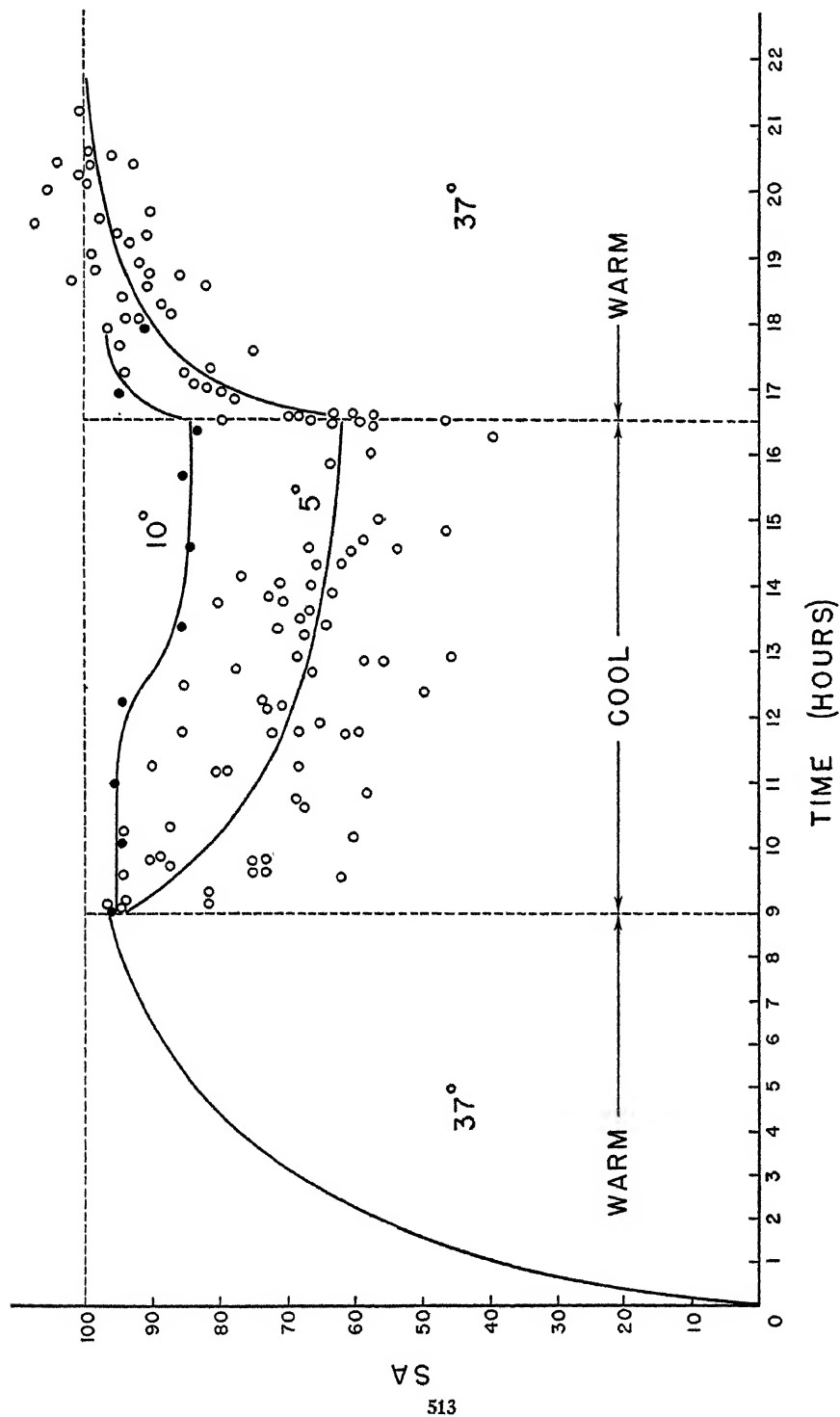


Fig. 1. Cold loss at 10° and 5° and recovery on restoration to 37°, from pooled data. Cultures first brought to approximate equilibrium with radio-

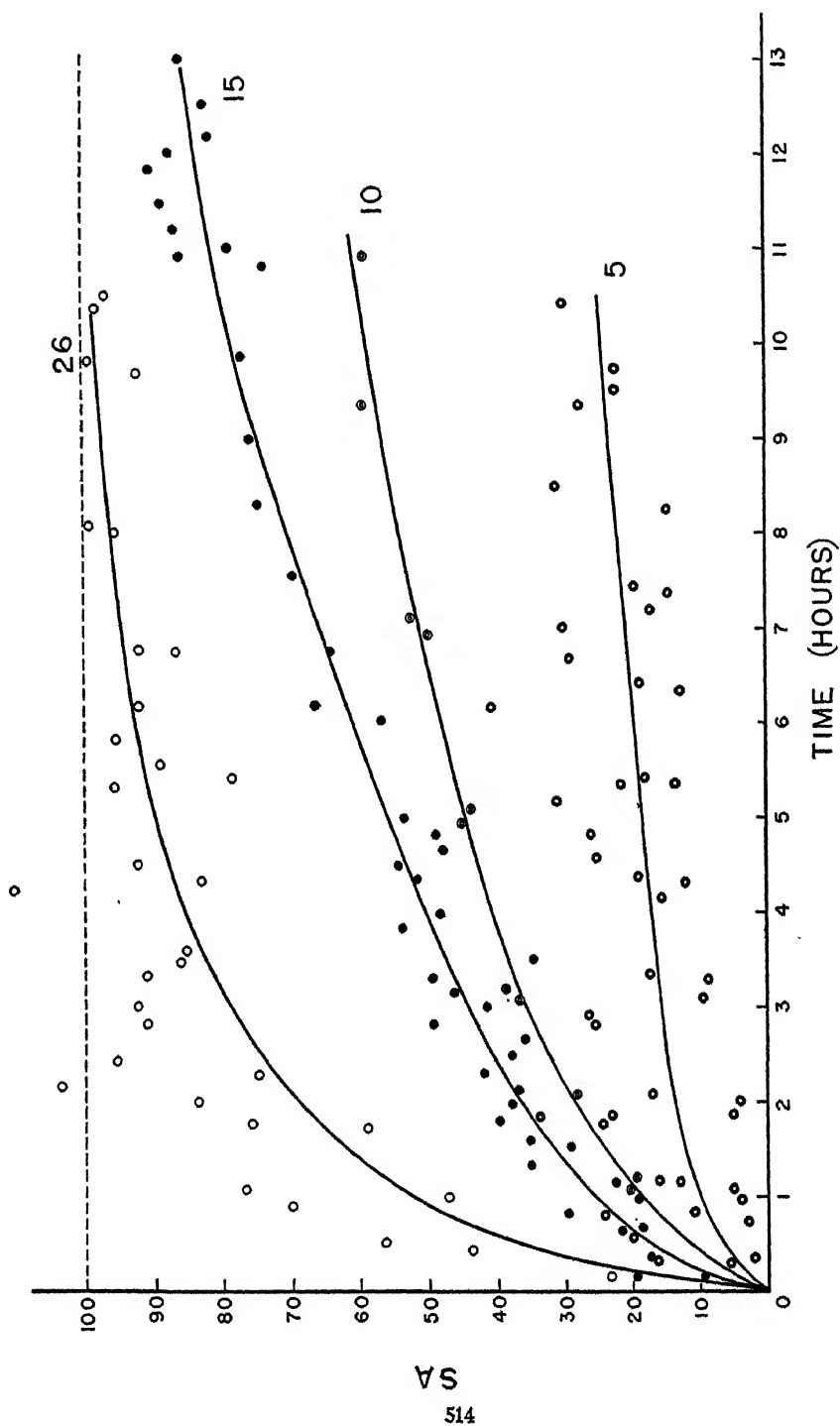


FIG. 2. Uptake of radioactive potassium by chick embryo muscle at 26°, 15°, 10°, 5°, drawn from pooled data. 26° (○); 15° (●); 10° (○); 5° (●). The curves at 10° and 5° are given in terms of the amount of potassium present in the cells at 37°.

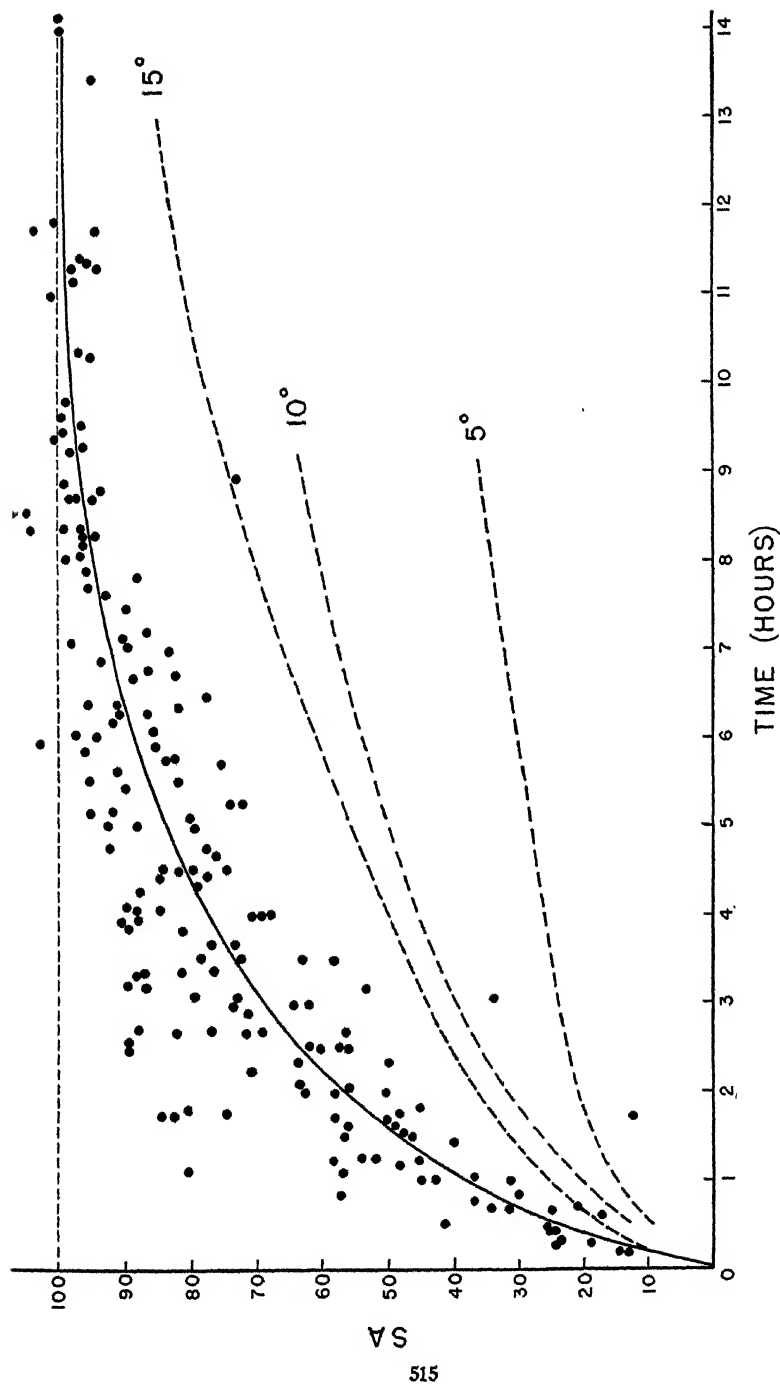


FIG. 3. Uptake of radioactive potassium at 37°, drawn from pooled data. Broken lines represent uptake curves at 10° and 5° after correction has been made for cold loss.

amounts present within the cells at the lower temperatures as estimated from the data on cold loss (Fig. 1). If correction is made for estimated cold loss so that the uptake curves reflect the approximate rate of attainment of equilibrium of the potassium which is actually present in the cell at the lower temperature,

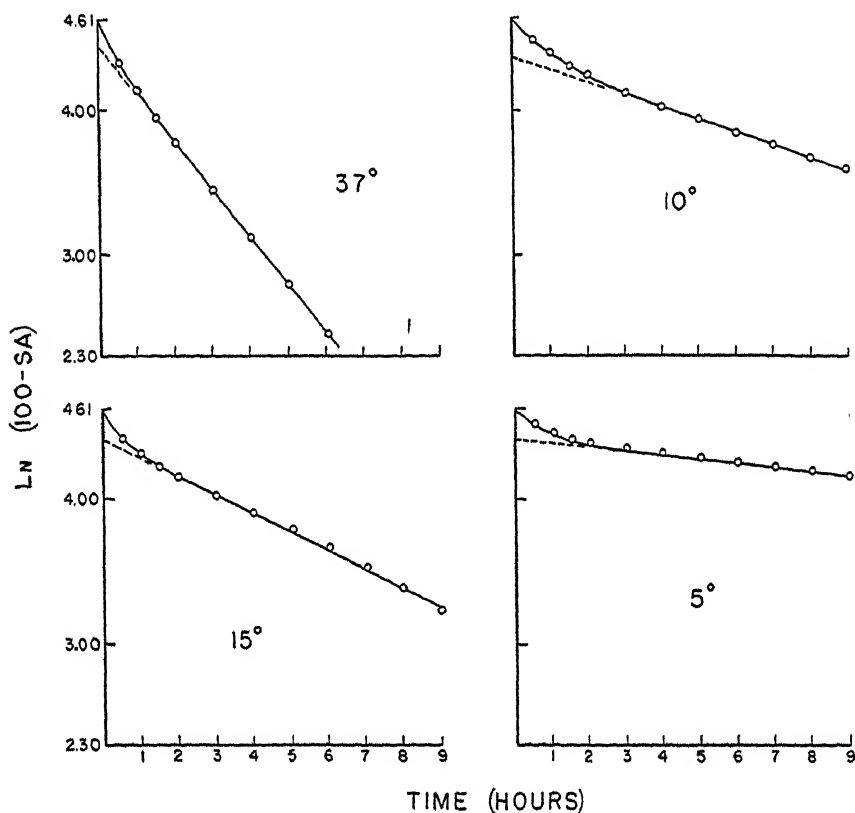


FIG. 4. Logarithmic plots of the uptake curves at 37°, 15°, 10°, 5°. Plots at 10° and 5° are drawn from the curves in Fig. 3. The linear portion is extrapolated to $t = 0$ (broken line).

the curves in Fig. 3 (broken line) are obtained. The uptake curves at 15° and at 37° drawn from pooled data are included in Fig. 3 for comparison. No significant difference was found between the rates of uptake at 37° and 26°.

The potassium uptake curves illustrated in Fig. 3 are plotted semilogarithmically in Fig. 4. All curves are angulated, showing an initial portion with a large slope and a later portion with a smaller slope.

In a system in which an ion behaves as a single molecular species, the uptake

of an introduced radioactive tracer should behave according to Fick's equation, which in this case takes the form (2):

$$SA = 100 (1 - e^{k \cdot P/P_s \cdot t}),$$

where k = turnover rate, or proportion of cell potassium exchanged per hour,

t = time, in hours, and

P/P_s = ratio of total potassium to that in the medium, which, as determined under the present experimental conditions, is approximately 1.15.

The factor $k \cdot P/P_s$ can be obtained from experimental results by plotting $\ln(100 - SA)$ against time. If Fick's equation describes the data, this semilogarithmic plot should be a straight line, with $k \cdot P/P_s$ equal to the negative slope. Our data as plotted in Fig. 4, do not, however, yield such a straight line. This suggests that two potassium components are involved, one of which (P_d) turns over much more rapidly than the other (P_n).

Two potassium components may be related in either of two ways: (a) P_d and P_n may be simultaneously in equilibrium with P_s ; or (b) only P_d may be in equilibrium with P_s , while P_n is in equilibrium with P_d . Case (b) is more complicated, and the mathematical equations which describe it have been calculated for phosphate (2), in which case the components can be estimated by chemical analysis, and where there is good reason to believe that the conditions are correctly described by it. It has been assumed here that the two hypothetical states for potassium are described by case (a), although there is no reason to believe *a priori* that this should be the case. In any event, direct measurement of specific fractions of cell potassium is not possible at present and calculations under case (b) can therefore not be made. Moreover, theoretical uptake curves according to either case (a) or case (b) may so closely resemble one another that either could fit the present data.

Assuming that exchange between P_d and P_n can be neglected as a rate-determining factor, a system containing two potassium fractions exchanging with the medium may be described by the following equation:

$$SA = 100 \left(1 - \frac{P_d}{P_i} \cdot e^{-1.15k_1t} - \frac{P_n}{P_i} \cdot e^{-1.15k_2t} \right) \quad (1)$$

where k_1 = turnover rate of the "fast" fraction, P_d ,

k_2 = turnover rate of the "slow" fraction, P_n ,

$P_i = P_d + P_n$

P/P_s has been taken as 1.15, which is the approximate mean value under the experimental conditions. When k_1 is much greater than k_2 , and t is large, the first exponential term of equation (1) may be ignored, so that:

$$\ln(100 - SA) = \ln \frac{P_n}{P_i} - 1.15k_2t \quad (2)$$

From the graph of $\ln(100 - SA)$ against time (Fig. 4), the slope of the later portion of the curve equals $-1.15 k_2$, and the extrapolated intercept at $t = 0$ is equal to $\ln \frac{P_n}{P_i}$, whence the magnitudes of P_n and P_d may be derived. Differentiating (1), the slope of the curve at $t = 0$ is given by:

$$\frac{dSA}{dt} (t = 0) = 1.15 \left(\frac{P_d}{P_i} \cdot k_1 - \frac{P_n}{P_i} \cdot k_2 \right) \quad (3)$$

Since P_d , P_n , and k_2 are known, and $\frac{dSA}{dt}$ may be estimated graphically, k_1 can be calculated.

TABLE I

Per Cent of Tissue Potassium in the Slow (P_n) and Fast (P_d) Fractions and Their Estimated Turnover Rates at Various Temperatures

Values are calculated from Fig. 4 and Equation 3.

Temperature	No. of cultures	P_n	P_d	k_2	k_1	No. of cultures	Per cent lost in cooling from 37°
°C.							
37	16	84	16	0.29	1.5	—	—
15	3	81	19	0.11	2.0	2	0
10	2	78	22	0.075	1.2	2	11.5
5	5	85	15	0.026	1.1	9	36.0

P_n = slow fraction, per cent of total.

P_d = fast fraction, per cent of total.

k_2 = turnover rate of the slow fraction per hour.

k_1 = turnover rate of the fast fraction per hour.

The size and rate constants of the fast and slow fractions as determined from the curves in Fig. 4 and calculated from Equation 3 are summarized in Table I. The rate constant of the fast fraction appears to change little with temperature whereas the rate of the slow fraction increases by a factor of 10 or more between 5° and 37°.

DISCUSSION

The data presented in this paper strongly support two conclusions: that potassium is lost from cells of chick embryo muscle on cooling; and that the rate of exchange of cell potassium has a large temperature coefficient. Quantitative values, the order of accuracy of which is still uncertain, can be given for both types of potassium movement. On the other hand, the interpretation of the observed deviation of the radioactivity uptake curves from the simple form of

Fick's equation is less clear. The following possible interpretations may be considered: (1) the initial rapid exchange represents an untraced experimental error; (2) the uptake curves represent exchange with two phases of potassium ion, differing in their rate of exchange with the radioactive isotope present in the medium. With regard to the first possibility, there is little that can be said. Contaminating sodium was probably eliminated by two reprecipitations of the radioactive potassium chloride from concentrated sodium perchlorate solution. The analysis of the data according to the assumption that two different potassium fractions exist in the tissue cultures has been presented above. Although the existence or the location of cell membranes is not essential to this analysis, the bulk of the tissue potassium and probably all the slow fraction are intracellular. Part of the fast fraction is probably extracellular, representing incomplete exchange of the potassium of the interstitial fluid with that of the medium in the bottle. Until uncertainties regarding the existence and nature of the fast fraction can be clarified, further conclusions will be hazardous.

There is little information in the literature on the temperature coefficient of the rate of uptake of radioactive potassium, which the present study shows to be quite large. Overstreet and Broyer (7) using radioactive potassium calculated that 10 per cent of the potassium in their "low salt" barley roots was exchangeable in 3 hours at 0°C. This was compared with a rapid and complete exchange at higher temperatures. In this respect potassium is analogous to phosphate, which penetrates erythrocytes little or not at all at 7°, but enters rapidly at higher temperatures (8). Cardiac muscle also shows a large temperature coefficient for the uptake of phosphate (9), and the movements of this ion are in general believed to be closely related to cellular metabolism (10).

In the case of phosphorus, many compounds are known whose turnover is linked with cell metabolism. Little is known, however, concerning the existence or nature of intracellular potassium compounds.

It is possible that the loss of potassium from cells at low temperature is a general phenomenon. Several investigators have shown that erythrocytes lose potassium to plasma when stored at 4–7°C. (11–14). The loss is independent of the previous history of the blood, and amounts to about 30 per cent of the cell potassium in a few days (11, 12). The lost potassium is partially regained on rewarming the cells. Conway and his coworkers (15) report that frog muscle loses 50 per cent of its potassium at 3° although they do not state how rapidly this loss takes place. Krogh (16), however, reports that although the chick chorionic membranes which he studied lost potassium at 3–7°, the calculated rate of loss was very slow, being equal to or less than 1 microequivalent per gram of wet membrane per hour. Here also potassium and phosphate behave in a similar manner, for Halpern (17) reports that phosphate is lost from erythrocytes at 3°C. and reenters the cells at 37°.

Thus the intracellular potassium of tissue cultures, like phosphate ion, has a turnover rate which is highly sensitive to temperature changes. It exchanges very slowly with the potassium of the medium at 5°, and rapidly at 37°. A considerable portion of it is no longer held in the cell at reduced temperature, but the loss is not proportional to the temperature, for none appears to be lost at or above 15°. Further, there is evidence that it may be divided into two fractions: the larger fraction having a high temperature coefficient and a slow turnover rate, the smaller having a low temperature coefficient and a more rapid turnover rate. Brooks (18), working with paramecia, found similarly that radioactive potassium exchanged most rapidly at the beginning of an experiment. He felt that this was due to "successive gains or losses of K^{++} " during the experiment. Nevertheless, available data are equally consistent with the possibility that we are dealing with a system in which the potassium is present in the cell in two different chemical states.

Two opposing theories concerning the nature of the concentration of potassium ion within cells are widely held at the present time. One of these is the theory that intracellular potassium is largely "bound," the other is that the intracellular potassium is "free" but that losses due to outward diffusion of intracellular potassium are repaired by a constant active transport of the ion inward again by the cell. Neither theory is yet wholly satisfactory.

The concept of intracellular binding of potassium has appeared in both the American and European literature for many years, and has been restated recently (19-22). According to these authors, a large fraction if not most of the intracellular potassium should be considered as being present in chemical combination with non-diffusible organic elements of the cell, or more generally, has a very low apparent activity coefficient. A relatively small portion is considered to be freely ionized, or with an activity coefficient approaching that in a simple aqueous solution of the same concentration. The "bound" potassium is in equilibrium with the "free" form, explaining the ready exchangeability with radioactive isotope. This theory ignores the difficulty of conceiving the nature of the osmotic equilibrium of animal cells between protoplasm and environment when the bulk of the potentially osmotically active intracellular substance is rendered inactive by binding. Our data accord well with the "bound" potassium hypothesis, and, considered *in toto*, seem difficult to explain in any other way. At 5°, the membrane is freely permeable, as is evident from the continued outward mass movement of potassium across the cell membrane for several hours after the cell has been chilled. Nevertheless, 50 per cent of saturation with radioactive potassium has not been reached in 12 hours. One could explain this either by postulating the existence of a group of intracellular organic potassium compounds whose dissociation constants fall rapidly with temperature, or by a decline in the rate of metabolic turnover of certain organic potas-

sium compounds. It may be supposed further that some of these organic potassium compounds require metabolic activity to maintain their stability, and hence are degraded at low temperatures, resulting in loss of intracellular potassium.

The alternative viewpoint has recently been expressed by Krogh (23) and Hald *et al.* (24). They consider that the potassium within cells is free, and that its concentration there is not an equilibrium but a "steady state." The cell must continually expend energy in order to transport potassium to replace that lost by outward diffusion. Similarly the cell is kept relatively free from sodium by an outward transport of that ion. The picture has been simplified by Dean (25) who shows that, theoretically, an outward transport of sodium alone can account for potassium accumulation. The observation that cooling produces a loss of cell potassium accords well with this theory, since it may be presumed that at lower temperatures the depressed cell metabolism no longer supplies sufficient energy to continue potassium or sodium transport. The low rate of exchange of added isotope with the remaining cell potassium at low temperatures is more difficult to explain. One must assume that the membrane becomes less permeable at low temperatures or else that the barrier to diffusion which it offers has a high energy coefficient. Either possibility is difficult to reconcile with the observed rapidity of outward movement during cold loss. Also difficult to reconcile is the non-linear logarithmic slope of the radioactivity uptake curve, suggestive of the possible existence of two intracellular potassium fractions with different rates of exchange.

SUMMARY

The effect of temperature upon the exchange rates between intra- and extracellular potassium in chick embryo muscle was determined by the use of radioactive potassium. The temperature coefficient of at least four-fifths of the cell potassium is large. At temperatures below 15°C., potassium is lost from the cell and is regained on warming. The results suggest the possibility that 20 per cent or less of the cell potassium may differ from the rest by being more rapidly exchangeable with the medium.

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PHOTOOXIDATIONS IN PIGMENTED BLEPHARISMA

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Blepharisma undulans possesses what appears to be a unique intracellular pigmentary system which sensitizes it to light. Some features of the effects of light on this form have been already explored (Giese, 1938, 1946): only well pigmented animals are killed and only by high intensities of visible light. Colorless animals, bleached by long exposure to light of low intensities, are not. The colored animals exposed to light after careful exclusion of oxygen are also not killed. These experiments suggested that killing is the result of photooxidation. If the process were a photooxidation, it should be possible to demonstrate an increase in oxygen consumption when a culture of *Blepharisma* is illuminated. Such proves to be the case as demonstrated in this report.

Materials and Methods

Blepharisma was grown in the manner previously described (Giese, 1946). Animals from vigorous cultures were concentrated by centrifuging gently at about 10 × gravity. They were pipetted into a Cartesian diver of a special design to give increased sensitivity and operated with a modified technique (Zeuthen, 1948). One diver was used for the entire series of experiments reported here. The diver was kept balanced or nearly balanced at all times; therefore it remained in view of the horizontal microscope and observations of the condition of the animals could be made at any time desired. In other words the gas volume was kept constant, the pressure being regulated by a manometer. In these experiments the blepharismas were kept under almost constant observation. The diver was made so as to float with the bulb upwards as shown in Fig. 1. This is desirable in any experiments where the animals are likely to be injured because even if they were to fall to the bottom of the culture medium containing them, they would not be removed from the source of oxygen since they would come to lie in the meniscus between the air bubble and the water. The air bubble is just big enough to effect a complete separation between the culture medium containing the animals and the alkaline neck seal (in several different ways, described later, we have made certain that there is no leakage between the two). As the animals swim in the diver they stir the medium continuously thus aiding the process of diffusion and making an adequate supply of oxygen available to animals removed from the meniscus.

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† On leave from the University of Copenhagen, Denmark. This work was performed during tenure of a Rockefeller Foundation Fellowship.

In a few of the early experiments the diver was filled with tap water and the blepharismas were pipetted into this. The blepharismas remained healthy in the diver, but in all subsequent experiments the diver was filled with culture medium which contains balanced salts and a 0.005 M phosphate buffer at pH 7.0 and is more constant in composition. Then a 1.10 μ l. air bubble was pipetted into the diver displacing the fluid, leaving about 100 blepharismas in about 1.5 μ l. in the bulb of the diver. Next the neck fluid was made alkaline with 0.1 M NaOH. The glass stopper which

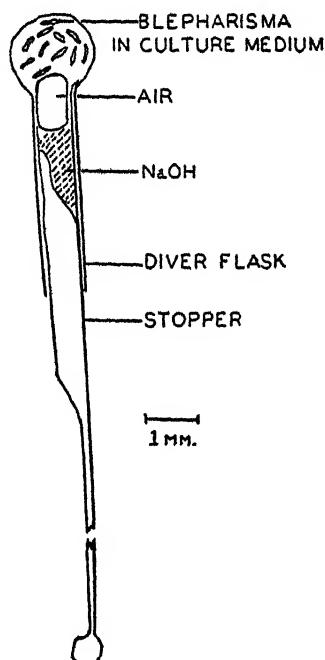


FIG. 1. The special Cartesian diver used in the experiments. In most of the experiments a longer tail was used. The diver bulb measured 1.75 by 0.91 mm. The air bubble was 1.43 mm. long, the stopper was about 8.60 mm. long of which 3.50 mm. was inserted into the flask. The inner diameter of the neck of the flask was 1.06 mm.

prevents diffusion of gases from the flotation chamber to the diver was then inserted and the diver pipetted into a flotation chamber filled with 0.1 M NaOH.

Before proceeding with the experiments it was necessary to study the behavior of an empty diver as a control before, during, and after illumination. An "empty" diver is filled with the same solutions as used in experiments but it is without animals. If the light or the heat generated by absorption of light were to produce large pressure changes, the diver would not be suitable for photobiological experiments. Fortunately these pressure changes are small and actually in reverse of the changes produced by respiration. That is, light causes a pressure increase in the gas space whereas respiration resulting from oxygen consumption results in a decrease in pressure in the

gas space. This is shown in Fig. 2. The beginning and the end of illumination are shown by dotted lines. These changes were even more strikingly shown when the diver was filled with India ink which absorbs almost completely the light falling upon it. In this case the excursions were of much larger order but still in the same direction as in the control. In both cases it was noted that shortly after the light was extinguished a pressure decrease about equal to the pressure increase at the start of illumination occurred. This may be seen in Fig. 2. We may conclude that the changes in pressure caused by the heating of the diver were so slight as to be negligible in

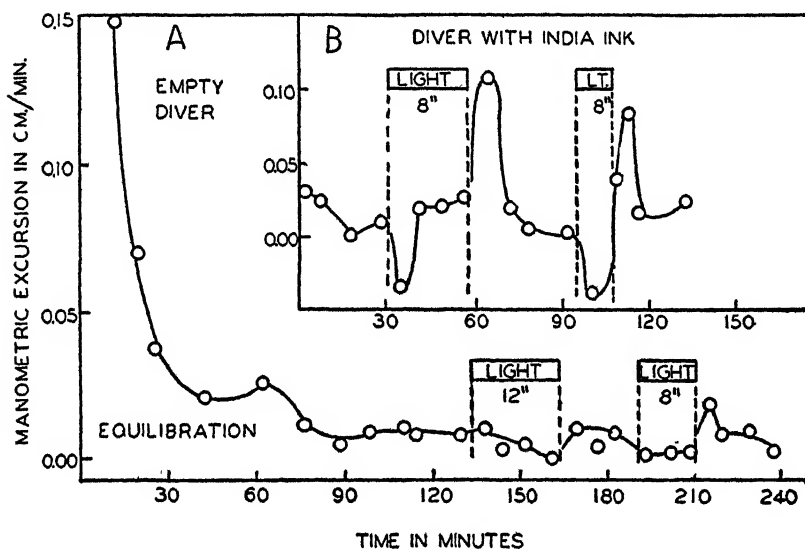


FIG. 2. In A are shown the period of equilibration and the effects of brilliant illumination on an "empty" diver. The diver in this case is filled with the solutions such as are used in experiments but lacks the animals. In B the diver used was filled with Higgins India ink. This gives a very strong absorption of light exaggerating photic effects. In addition, in order to exaggerate the effect the light was moved as close as possible—8 inches from the diver.

irradiation experiments where the changes are very large. Illumination of the compensation bottle or other parts of the equipment with intense light did not cause noticeable variations in the movement of the diver. Therefore stray light of low intensity ordinarily falling on parts of the equipment other than the diver and diver chamber, does not vitiate the experiment.

Fig. 2 shows another point of interest in technique. It will be noted that the curve falls rapidly from the initial reading until about 30 to 40 minutes later. This is the period of equilibration during which the gas in the air space and the fluid in the diver are coming into equilibrium. Such a period was always observed both in the diver with and without animals. All the experiments were performed after this period although the changes were recorded and plotted.

For illumination a G. E. 100 watt mercury spotlight was used, a voltage regulator-transformer serving as a source of power. This lamp gives a powerful spot of light. Since considerable heat is also present it is necessary to use a filter interposing at least 4 inches of water. In practice a heat-absorbing $m/40$ CuSO_4 water cell about 3 inches thick was used. At the same time this cell reduces the intensity of light by about 31 per cent. In addition to this filter the water in the water bath in which the diver was contained served to dispel the heat. In some experiments a $\frac{1}{2}$ inch cell containing 10 per cent NaNO_3 to cut out long ultraviolet radiations was used in addition. This reduces the light intensity by about 13 per cent. In all cases unless otherwise noted a Corning No. 3389 filter which reduces the light by about 35 per cent was used. When all three filters are used together the light intensity is reduced to about one-half of the original intensity. The Corning filter No. 3389 has a cut-off at 4100\AA ; therefore in all cases the cells were exposed to visible light only. The intensity of the light was determined with a thermopile calibrated against U. S. Bureau of Standards standard lamps. The intensity was of the order of $1000 \text{ ergs/mm}^2/\text{sec}$. at a distance of 16 inches. In most of the experiments the lamp was kept at 12 inches and in a few at 8 inches. The intensities might be calculated on the basis of the inverse square law but this is a rough estimate since the light source is large. Using a Western photronic cell the increase in intensity of the light source from 18 to 16 to 12 to 8 inches is indicated to be: 1:1.2:2.2:3.7.

The experiments were done in a thermostat kept at a relatively constant temperature by running sea water. The temperature generally remained constant for several hours; on some days a slight drift of 0.1°C . per hour occurred as the sea water in the storage tanks warmed up during the day. Such slight changes did not interfere with the experiments. A slight drift is preferable to a standard temperature bath in which alternate heating and cooling produce oscillations which set up disturbing convection currents in the flotation vessel. The temperature for the entire period of experimentation varied between 14.3 to 17°C . as the ocean water warmed up during the summer months.

The light in the room in which the experiments were performed was reduced to a minimum by drawing the shades. Since light of even much higher intensity than that of the diffuse light of a room so darkened seems to have little effect on the oxygen consumption of *Blepharisma* (see Fig. 3), these precautions were considered adequate.

EXPERIMENTAL

The increase in oxygen consumption of *Blepharisma* on illumination was apparent from the very first trials made. However this was observed only when the intensity of the light was high. When by the use of screens or by movement of the lamp the intensity of the light was reduced to $\frac{1}{8}$ no measurable increase in oxygen consumption was observed. When the screens were removed, however, a striking and unmistakable increase of oxygen consumption was observed, after which it fell. During this period of rapid consumption the animals were injured, died, and burst, liberating their contents. The data for one of the experiments are graphed in Fig. 3.

If the animals were illuminated for a long time at a light intensity lower than that at which injury or death occurred, they showed signs of bleaching. When the light intensity was subsequently raised, the oxygen consumption increased some, but not as strikingly as described above. In some experiments bleaching, on exposure to high intensity of light was observed, and, by the time death occurred, it seemed complete. In other experiments little bleaching was observed. Unfortunately it was not possible to have a continuous supply of ani-

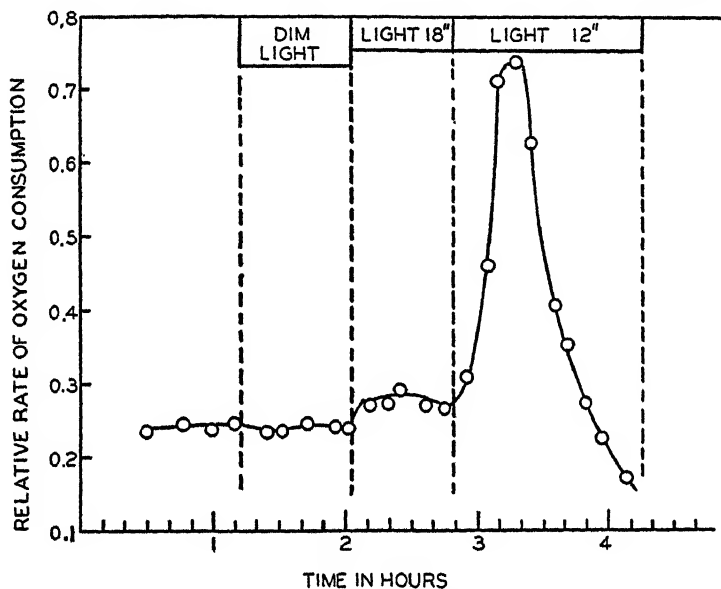


FIG. 3. The rise in oxygen consumption on bright illumination (12 inches) of a diver filled with highly pigmented blepharismas. Relative rate of oxygen consumption is given in centimeters excursion of manometric fluid per minute. Absolute values could be determined from these values but in absence of precise data on the amount of protoplasm involved this was not considered worthwhile. "Dim light" is the light of a Spencer universal microscope lamp No. 353.

imals of the same degree of coloration. The amount of pigments present at the beginning and perhaps other factors such as the age of the culture employed may account for these differences in behavior.

If the pigment in the cells is the photodynamic sensitizer as is indicated by the fact that bleached blepharismas are not killed when exposed to intense visible light (Giese, 1946), then illumination of the bleached cells should not result in an increased oxygen consumption. In Fig. 4 are shown the results of illumination of partially bleached animals (24 hours' exposure to the light of a 6 watt daylight fluorescent bulb) and in Fig. 5 for rather thoroughly bleached animals (48 hours' bleaching). The results indicate that when some pigment

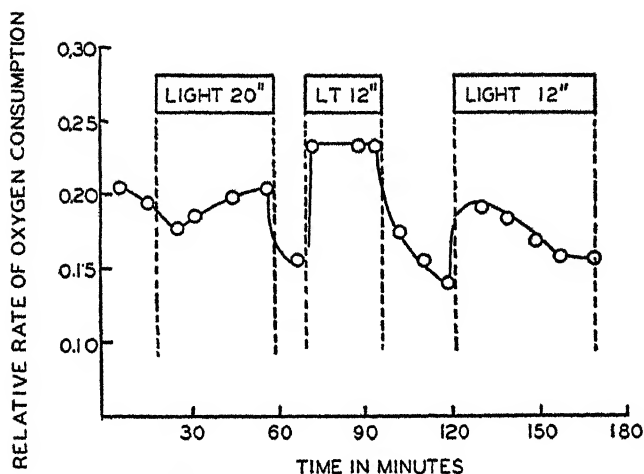


FIG. 4. Increased oxygen consumption of partially bleached blepharismas (bleached for 24 hours). Rate measured as in Fig. 3.

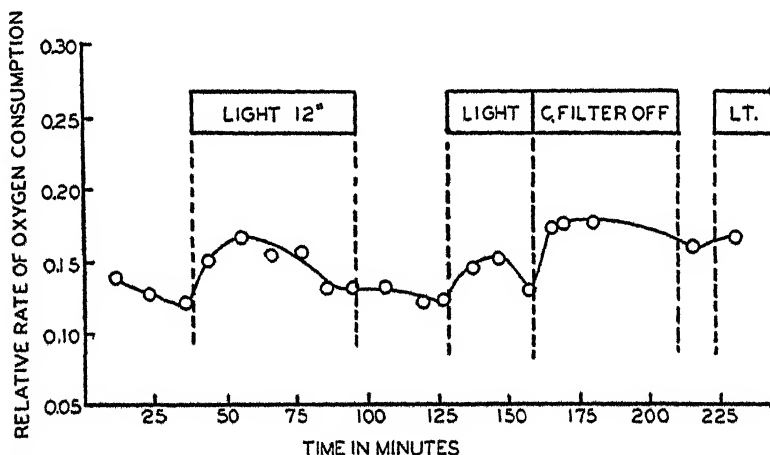


FIG. 5. The increase in oxygen consumption of relatively bleached blepharismas (bleached 48 hours). Rate measured as in Fig. 3. The "C" filter referred to is the Corning No. 3389 filter which cuts out the ultraviolet radiations. Stimulation following removal of this filter may be due to increased intensity of light or to the long wave length ultraviolet added.

is present, as in the partially bleached cells, a slight increase in oxygen consumption is obtained (Fig. 4); however when bleaching is more thorough, even intense light has only a very slight effect (Fig. 5).

Even though the bleached cells are not injured by intense light they show some signs of stimulation by the light. Thus, if they have become sluggish and

have settled on the meniscus, light stimulates them to more rapid swimming, and a small increase in oxygen consumption is found.

An even more dramatic demonstration that *Blepharisma* is not injured or killed by light except in the colored state is demonstrated by irradiating a mixture of the bleached and colored individuals. Illumination brings about rise in oxygen consumption and only the colored animals are injured and killed. As they cytolyze they leave behind only the bleached animals which seem to be unaffected by the fragments of their colored fellows. The behavior of the bleached individuals is apparently not much affected by the presence of any of the materials released by the cytolysis of the colored ones.

At the beginning of the research the question arose as to whether the alkali might perhaps creep as a result of illumination, killing the animals and causing the rise in oxygen consumption. Since illumination of bleached animals results in only a small increase in oxygen consumption without injury to the animals, there could have been no creeping of alkali. The selective killing of the colored animals, when mixtures of the colored and bleached ones were exposed to light, is further confirmation of the effect of light not alkali.

Is the increase in oxygen consumption due to acceleration of the respiration of the animals or is it due to initiation of photooxidations extraneous to life? If it is due to action on the enzyme systems then it should not show up on illumination of animals killed by heat. If it is due to photooxidations extraneous to life, the increase of oxygen consumption should show up even with heat-killed cells, so long as the pigment is present. To test this, a concentrate of animals was immersed in boiling water for one-half minute. The red bodies of the animals and the pinkish fluid were pipetted into the diver. The preparation in the dark showed no respiration, as might be expected. The very small excursions of the manometric fluid due to minor changes in pressures were observed, comparable in magnitude to those of a diver devoid of animals (compare to Fig. 2) showing that the pigment carried on no oxidation in the absence of light. Illumination was followed by an increase of oxygen consumption like that found with the colored living animals. This rise is followed by a rapid decline, then a much slower decline, as seen in Fig. 6. Since the curve shows a rather striking break, it was thought at first that two reactions were involved, the first being the oxidation of the dyestuff in solution, the second of that in the corpses or *vice versa*. An attempt was made to test the latter possibility by using only the supernatant fluid after a heat extraction. Since the same sort of curve is obtained in this case as well, the simple explanation obviously does not hold. The slow oxidation in both cases may be one of the secondary reactions after the pigment first reacts with oxygen.

When the light striking the solution of the pigment is suddenly intercepted, the rate of oxidation falls quickly to a very low value. Illumination again is followed by a rapid rise of oxygen consumption. The oxidations are therefore

most likely photooxidations, not merely initiated by light, but dependent upon light for their expression. Since it is impossible to work in a completely darkened room, the low oxidations continuing when the intense light is cut off may be due to the small amount of light reaching the pigment from the diffuse light in a room darkened by drawing ordinary window shades.

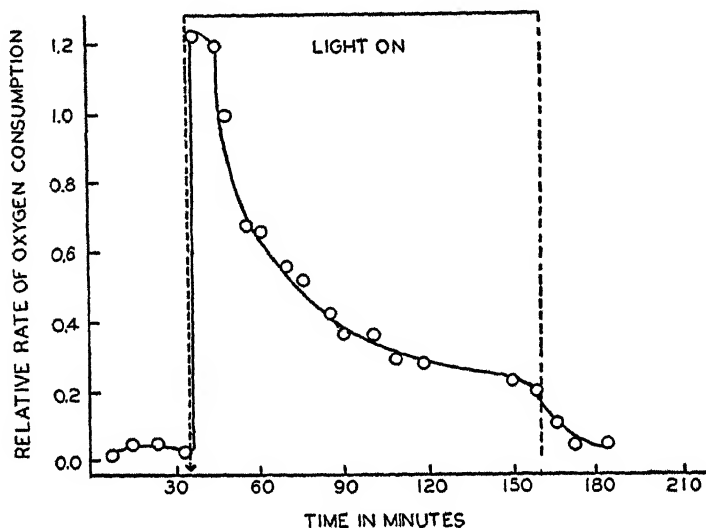


FIG. 6. The oxygen consumption of a suspension of dead bodies and a solution of pigment extracted from a concentrate of animals immersed in boiling water for half a minute. Note that the curve shows a rapid drop for a short time then a much slower decline suggesting two separate reactions. Rate of oxygen consumption measured as in Fig. 3. It is probable that the number of bodies is much greater than the number of live animals pipetted into a diver since it is much easier to pick up dead ones. Unfortunately they were not counted.

Since pigment extracted by heat treatment might contain protein in addition to the colored material, extraction by alcohol was resorted to. This should get rid of most if not all the protein since only the plant proteins known as gliadins are soluble in alcohol (80 per cent). A concentrate from a blepharisma culture was therefore extracted with absolute ethyl alcohol, the solution was dried by slight heating in a water bath, and the dried material was redissolved in absolute alcohol. It was put in the diver and an air bubble added in the usual way. The diver chamber was filled with absolute alcohol and after equilibration the solution was illuminated. A rise of oxygen consumption was observed also in this case. This indicates that the pigment in this state will photooxidize. Cessation of illumination stops the oxidation. In another experiment the diver was filled with concentrated eosin which acts as a photosensitizer in general and illuminated. Measurements indicated an oxygen consumption during illumina-

tion. The pigment from *Blepharisma* therefore resembles eosin in this respect at least.

Two variables which were not adequately studied might be fruitfully pursued: the effect of pH and the depth of coloration of the animals on the course of the reactions. The two seem to be interrelated to some extent. The depth of coloration appears to be largely a function of age of the population, since a vigorous rapidly increasing culture is generally light in color even though it is kept in the darkness. Only as the bacterial food is depleted and the static state of a culture is achieved does the deep red color develop. Although pigment acts as a sensitizer and although presumably more pigment per unit protoplasm would appear to be present in the deeply colored animals, their resistance to light is greater than that of lighter individuals from an active culture. There is also a greater spread in sensitivity in the older population, an observation not uncommon in studies on resistance of protozoan and bacterial populations to various factors. The pH of a culture is another variable of importance. Animals from an older culture resist light exposure about twice as well when placed in fluids buffered at a low pH (5.1) as at a higher pH (8.0). This is especially true if carbon dioxide is first bubbled through the droplet or bubbling continues at a low rate during exposure. These relationships can probably be studied more readily by techniques other than the Cartesian diver. However one observation with the diver should be mentioned here as pertinent to the discussion. When the alkali was omitted from the diver in a few experiments, it was observed that the increases in oxygen consumption and the injurious effects of light were much less striking especially in old cultures.¹ In the diver under these conditions carbon dioxide is bound to accumulate and the pH of the slightly buffered (0.005 M phosphate) culture medium may change. This change may protect the animals from the radiations.

DISCUSSION

The present work demonstrates that upon illumination of colored blepharismas, a great burst in oxygen consumption occurs. During the period of greatly increased oxygen consumption the animals are killed and cytolyzed. Only a small increase in oxygen consumption occurs if the animals are first bleached, the increase being greater the less the degree of bleaching.

Experiments show that even if the animals are first killed with heat, illumination of their corpses and the fluid in which they were heated brings about a large oxygen consumption. The increased consumption of oxygen is therefore not due to an effect on the enzymes but to oxidations extraneous to life. In fact it will occur in the aqueous extract (heat) or alcoholic extract of the animals in the absence of their bodies. Cessation of illumination is followed by a great drop in the oxidation rate. Re-illumination again raises it. The oxidations are therefore photooxidations occurring during the illumination of the pigment(s).

¹ Even under these circumstances the animals may be killed by very strong light.

The phenomenon seems in general to resemble the photodynamic effects of various fluorescent dyes, except that in *Blepharisma* the dye is present in the cell. Thus Wohlegemuth and Szorenyi (1933) state that blood cells and tissues showed an increased "respiration" during illumination, if a photodynamic substance was present, and that this increased oxygen uptake was due to the extraneous photooxidation of cell materials since even boiled cells showed the same phenomenon. This, however, was not found to be the case in yeast (Freeman and Giese, unpublished). It would seem that the photooxidation produces something which is harmful to the cells. This is surmised from the fact that the division of cells continuously subjected to weak light is retarded (Giese, 1946). However the injury is not very serious since the cells go on dividing and under appropriate conditions they conjugate (Giese, 1938). As shown in the present work partially and even rather completely bleached cells show some increased oxygen consumption and they move about more actively after illumination yet even intense light does not kill them. Whatever injury occurs is insufficient to produce visible effects. Even deeply pigmented cells subjected to less than the full intensity of light may show increased respiration without visible injury. It would seem that only when the formation of the photooxidative products reaches a certain rate are the cells adversely affected.

The photodynamic dyes such as eosin may act in either of two ways: (1) the pigment might be attached to a protein. On becoming activated by absorption of light energy, it transfers its energy to the protein, which then reacts with oxygen; (2) the pigment itself might become activated and oxidized and thereby acquires the ability of oxidizing the protein. The protein breakdown, however it occurs, is considered the fatal process (Blum, 1941).

The experiments on *Blepharisma* show only that an oxidation is involved in killing by light as indicated by the increased oxygen consumption in bright illumination, and while some support for each of the two possibilities, listed for the mechanism of action of photodynamic dyes in the paragraph above, may be found in the experiments on *Blepharisma*, the data do not permit a choice between them. Thus the pH sensitivity of the killing by light suggests the first possibility, namely a union of pigment and protein but it may also be explained by the dissociation of the dye molecule itself. Also the fact that the bleached animals are unharmed when irradiated in a mixture with a high concentration of colored ones, even when the latter burst and liberate their contents, might be interpreted as indicating a non-penetrating toxic proteinous complex with the dye. However, the bleached animals may survive merely because the toxic substances liberated by the colored ones never reach the threshold concentration in the surrounding medium necessary for injury and not because these substances fail to enter. The fact that the pigmented animals must be distinctly reddish to be affected by light indicates the importance of the concentration factor. On the other hand the second possibility above is supported by the fact that the colored protein-free alcoholic extracts of *Blepharisma* take up oxy-

gen on illumination, indicating that something other than the protein, and perhaps the pigment itself, is oxidized in the light.

The presence of pigment in a cell does not necessarily mean that it will be photosensitive. Thus *Holosticha rubra* while possessing a brownish red pigment is neither bleached nor injured by exposures to light of the intensity used on *Blepharisma* in the present instance (Giese, 1946). A similar negative result was found recently with *Fabrya salina*, a black brine pool ciliate. A study of the types of pigments and their attachment in the cells reacting in different ways might give further clues as to the way in which the pigment of *Blepharisma* produces its effects.

The significance of the pigment in the metabolism of *Blepharisma* is still without explanation. Experiments show that this pigment is oxidized in light. It may also be oxidized reversibly in the dark and constitute a useful redox system. In bright light, however, it oxidizes some vital cell constituent(s) resulting in death.

SUMMARY

1. *Blepharisma undulans*, a protozoan with a reddish pigment, shows increased oxygen consumption under the influence of light.

2. If the light intensity is high, the animals are killed during a burst of oxygen consumption.

3. If the blepharismas are first bleached by exposure to light of low intensity they show only slightly increased oxygen consumption under the influence of light and they are not killed.

4. A preparation in which the animals are killed by heat still shows the increase in oxygen consumption on illumination with brilliant light. The supernatant solution does so as well, as does an alcohol extract of the dye.

5. The conclusion is drawn that the blepharismas are killed during photooxidation of the pigment, but the mechanism of action is not clear. Several possibilities are considered in the discussion.

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STUDIES ON THE MECHANISM OF ACTION OF IONIZING RADIATIONS

I. INHIBITION OF ENZYMES BY X-RAYS

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The effect of ionizing radiations on enzyme systems has been the subject of numerous investigations. In the early experiments, however, the amount of radiation necessary to produce inhibition was so high that Scott (1) quite reasonably concluded that x- and gamma rays only influence enzymes when the dose is enormous. The reason for these failures may have been the use of large amounts of enzyme and of impure preparations. In fact, Dale in 1940 (2) by diminishing the concentration of the enzyme, carboxypeptidase, obtained inhibition on irradiation with relatively small doses of x-rays. Dale was the first to postulate that enzyme molecules are not directly affected by the ionizing radiation, but indirectly through collision with a labile product resulting from the ionization of water. This indirect action had already been suggested in 1930 by Risse (3) in his "activated solvent" hypothesis, and was later developed by Fricke (4). Ionizing radiations may act on the protein moiety of the enzyme or on its prosthetic groups. When acting on the protein moiety they may destroy selectively certain groups in the side chain of the molecule that are essential for enzymatic activity, or they may act by breaking hydrogen bonds with production of denaturation or precipitation. When x-rays act on solutes dissolved in water there may result a number of oxidations by the products of ionization of water, such as oxidation of sulfhydryl groups, among the enzymes that require their presence for activity. It is therefore reasonable to assume that these enzymes may be preferentially inhibited on irradiation through oxidation of their sulfhydryl groups to the disulfide. This inhibition would be reversible. If the irradiation dose were increased other groups on the protein might be attacked. In fact, Svedberg and Brohult (5) split hemocyanin into two halves under the action of radiation from radium. The experiments presented in this paper on the reversible inhibition of sulfhydryl enzymes on irradiation with x-rays, and further irreversible inhibition when the dose of x-rays is increased, favor the opinion that sulfhydryl enzymes are the most sensitive to the action of ionizing radiations.

In this, as in subsequent studies on the biological effects of ionizing radiations, we have purposely kept the dose below 5,000 roentgens. In our opinion

the mechanism of action of larger doses of x-rays is more complicated and has no biological interest.

EXPERIMENTAL

Extreme care was taken to have all the water used in these experiments copper-free. Three times distilled water was twice redistilled in all-glass distillation apparatus (pyrex), one containing acid permanganate, the other, barium hydroxide.

Phosphoglyceraldehyde dehydrogenase from rabbit muscle was prepared according to the method of Cori *et al.* (6). Recrystallization of the protein was accomplished by dissolving the centrifuged residue in neutralized 0.02 M glutathione and by addition of 2 volumes of saturated $(\text{NH}_4)_2\text{SO}_4$. The crystals suspended in $(\text{NH}_4)_2\text{SO}_4$ containing glutathione and kept at 3° maintained their activity for 1 month. Phosphoglyceraldehyde was prepared according to the method of Meyerhof (7). Some of the phosphoglyceraldehyde used in these experiments was kindly provided by Dr. Meyerhof. Diphosphopyridine nucleotide (DPN) was prepared by the method of Williamson and Green (8). It was 50 per cent pure. The enzyme suspension (1 cc.) was centrifuged at 3° in the high speed centrifuge (15,000 R. P. M.) for 30 minutes. The supernatant fluid was removed with a capillary pipette and the solid was diluted to the appropriate amount in either buffer or in Cu-free water. Na arsenate and Na pyrophosphate were highest purity Kahlbaum samples and were Cu- and Fe-free. Phosphate buffers were made from reagent grade Merck's phosphates. In every case the samples which acted as controls accompanied the irradiated samples to the place of irradiation and were kept at the same temperature. Tests of enzyme activity were performed in a Beckman spectrophotometer in quartz cells, at 25°, by measuring at 3,400 Å the rate of reduction of diphosphopyridine nucleotide in the reaction: phosphoglyceraldehyde + $\text{DPN}^+ \rightarrow$ phosphoglyceric acid + $\text{DPNH} + \text{H}^+$, which in the presence of arsenate goes to completion. The quartz cells contained 0.1 cc. of enzyme (7 micrograms); 0.3 cc. phosphoglyceraldehyde (4×10^{-7} M); 0.2 cc. DPN 2.5×10^{-7} M; 0.2 cc. Na_2HAsO_4 (6×10^{-6} M); 1 cc. of pyrophosphate buffer pH 8.5 (3.5×10^{-8} M); and water to a total volume of 3 cc. As found by Warburg and Christian (9), for the yeast enzyme when the amount of muscle enzyme was the rate-determining step in the reaction, an exponential curve was obtained on plotting the K^1 values at the end of 1 minute (Fig. 1). These K values were used for the measurement of enzyme activity.

Ribonuclease, five times recrystallized, was kindly provided by Dr. Kunitz. The activity of the enzyme was determined manometrically by measuring the amount of CO_2 produced in bicarbonate buffer, since the enzyme splits ribonucleic acid into smaller acids and thus increases the acidity of the system. Purified commercial nucleic acid (0.01 M) was used as substrate; it was dissolved in bicarbonate buffer pH 7.4, saturated with $\text{N}_2:\text{CO}_2$ (95:5). The enzyme was dissolved in water so that 1 cc.

¹ The K values as defined by Warburg and Christian are obtained from the equation $K = \frac{1}{t} \frac{C_0 - C}{C_0 C}$, where t is time in minutes; C_0 , the initial concentration of DPN, and C , the concentration of DPN at time t .

contained 2.5 micrograms. As can be seen in Fig. 2, the amount of CO_2 liberated was proportional to enzyme concentration from 2.5 to 10 micrograms.

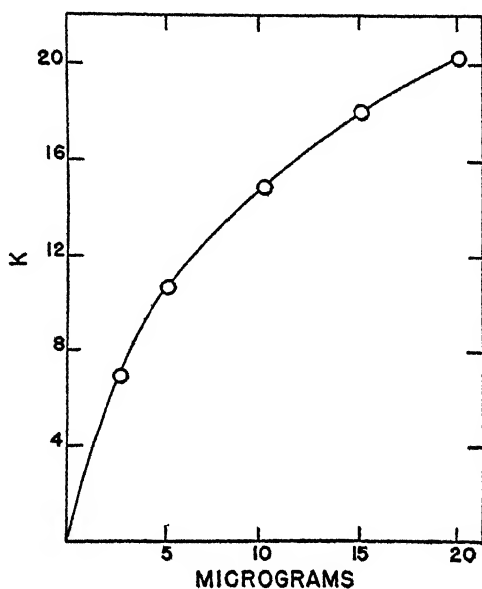


FIG. 1

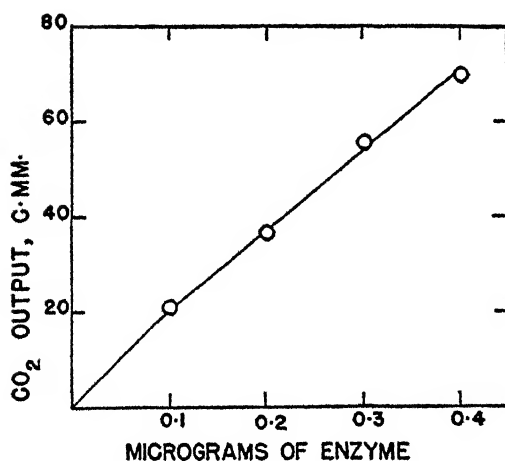


FIG. 2

Crystalline trypsin, three times recrystallized, was kindly provided by Dr. Kunitz. The activity of the enzyme was measured according to Anson (10). The conditions for the test were as follows: urea-hemoglobin substrate, pH 7.5, 2.5 cc.; enzyme in

water, 0.5 cc., or less; water to 3 cc. The samples were incubated for 10 minutes at 25°. Five cc. of 0.3 N CCl_3COOH was added and the samples were filtered 25 minutes later. To 2 cc. of the filtrate were added 4 cc. of 0.5 N NaOH and 1 cc. of the Folin-Ciocalteu reagent. The color produced was read after 5 minutes in the Coleman spectrophotometer at 6,300 Å.

Adenosinetriphosphatase (myosin) was prepared according to the method of Bailey (11). Ten to 40 micrograms per cc. was used in the irradiation experiments. Enzyme activity was determined by measurement of inorganic P after 10 minutes' incubation at 38°.

Succinoxidase from pigeon breast was prepared according to Barron and Singer (12), and enzyme activity was measured by the O_2 uptake on addition of succinate.

Lactic dehydrogenase from beef heart was prepared according to Straub (13). One hundred and sixty-three micrograms of this protein was used in the irradiation tests. Enzyme activity was determined by measurement of O_2 uptake on addition of DPN, flavoprotein, methylene blue, lactate, and cyanide. The purity of the protein moiety was 32 per cent.

Cytochrome oxidase was prepared from pig's heart by the method of Haas (14). Enzyme activity was measured by the O_2 uptake at 38° in the presence of cytochrome C (2.5 mg.); 0.3 cc. of 0.1 M phosphate buffer, pH 7.0; 0.3 cc. of 0.1 M hydroquinone and 1.2 cc. water.

Catalase was prepared according to Dounce (15). Enzyme activity was measured by titration of H_2O_2 with KMnO_4 at pH 6.8 and at 0°.

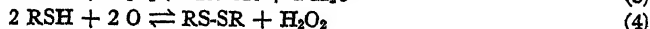
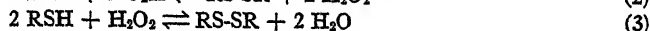
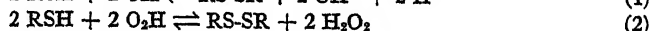
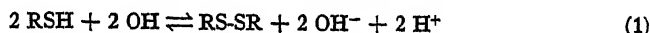
Irradiation was performed at the temperature of crushed ice with a 200 k.v.p. x-ray machine in a General Electric X PT tube (oil-cooled, air insulated). The enzyme solutions were placed in flat bottomed vials (20 mm. high X 16 mm. diameter) and placed in a depression of an aluminum holder which was set on cracked ice. The x-ray dose was measured with Victoreen condenser chambers inserted into the plastic container at the level of the solutions. The vials to be irradiated were placed on a raised plastic floor of a plastic container built with double walls so that cracked ice could be placed around and under the vials. This container was centered under the x-ray tube each time a run was made.

Effect of X-Rays on Sulfhydryl Enzymes

To test the effect of x-rays on the activity of sulfhydryl enzymes, the following sulfhydryl enzymes were chosen: phosphoglyceraldehyde dehydrogenase, which was shown by Rapkine (16) to require the presence of —SH groups for enzyme activity; adenosinetriphosphatase (myosin), which was shown to be a sulfhydryl enzyme by Singer and Barron (17), succinoxidase, demonstrated to be a sulfhydryl enzyme by Hopkins and Morgan (18), and hexokinase.

1. *Phosphoglyceraldehyde Dehydrogenase*.—For the irradiation experiments 70 micrograms of the crystalline enzyme was used, dissolved in phosphate buffer of different pH values, and of ionic strength, $\mu = 0.2$. The enzyme was irradiated with doses of x-rays varying from 25 r to 500 r. Half-inhibition occurred with 200 r, and almost complete inhibition with 500 r. If this in-

hibition was entirely due to the oxidation of the —SH groups of the enzyme by the products of water irradiation, (the radicals OH and O_2H , and the H_2O_2 and atomic oxygen), the following reversible oxidations could occur:



It would be possible to reduce the disulfides by addition of glutathione and thus restore enzyme activity. When the enzyme was 21 per cent inhibited, there was complete reactivation on addition of glutathione; when the enzyme

TABLE I

Effect of X-Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase from Rabbit Muscle

Enzyme (70 micrograms in 1 cc.) irradiated in phosphate buffer, pH 7.0 at 0°. Glutathione, 0.002 M. Enzyme activity, $K = \frac{1}{t} \times \frac{C_0 - C}{C_0 C}$ where t , time in minutes; C_0 (DPN) initial; C (DPN) at time t . Temperature 25°.

X-ray dose	Enzyme activity	Inhibition	Reactivation with glutathione
t	$k \times 10^4$	per cent	per cent
None	4.80		
25	4.80	None	
50	4.80	"	
100	3.81	21	Complete
200	2.43	50	62
300	0.98	80	
400	0.98	80	
500	0.25	94	10

was half-inhibited, there was 62 per cent reactivation; and when the enzyme was completely inhibited, reactivation was only 10 per cent (Table I). In these reactivation experiments enzyme activity was tested immediately after addition of neutralized glutathione (0.002 M). Cori and Cori (19) have pointed out, however, that a time factor is involved in the reaction between the enzyme phosphorylase and glutathione. Whether greater reactivations would have been obtained by allowing more time for complete action is not known.

When the pH of the solution is decreased, the concentration of H_2O_2 formed will increase because a high pH value favors a lower stationary H_2O_2 concentration (20, 21). Furthermore, the oxidizing power of the radicals OH and O_2H , as well as H_2O_2 increases as the H^+ ion concentration increases; hence —SH enzyme inhibition by ionizing radiations will decrease as the pH increases. The experiments in Table II show this to be the case.

If the inhibiting effect of x-rays on this enzyme is mainly due to oxidation

of —SH groups, it will be possible to protect the enzyme by previous addition of glutathione. The experiments in Table III show that previous addition of glutathione reduced the inhibition from 50 per cent to 9 per cent. Addition of diphosphopyridine nucleotide had no effect at all.

2. *Yeast Hexokinase*.—The enzyme hexokinase which catalyzes the reaction $\text{glucose} + \text{ATP} \rightleftharpoons \text{glucose-6-phosphate} + \text{ADP}$ has been prepared in crystalline form from yeast by Kunitz (22) and by Berger *et al.* (23). The crystalline yeast hexokinase was kindly provided by Dr. Kunitz. Hexokinase is an —SH

TABLE II

Effect of pH on the Inhibition of Phosphoglyceraldehyde Dehydrogenase by X-Rays
Buffer, phosphate; x-ray dose, 400 r.

pH	Activity		Inhibition <i>per cent</i>
	Control $k \times 10^6$	X-ray $k \times 10^6$	
6.2	12.2	3.86	63
8.3	16.6	10.4	32

TABLE III

Inhibition of Phosphoglyceraldehyde Dehydrogenase by X-Rays
Protection with Glutathione

X-ray dose, 200 r; pH, 7.0.

Experimental conditions	Activity	Inhibition
	$k \times 10^6$	<i>per cent</i>
Control.....	4.8	—
Enzyme treated with x-rays.....	2.43	50
“ “ + glutathione, $1.8 \times 10^{-3}\text{M}$	4.33	9
“ “ + DPN, $2.4 \times 10^{-4}\text{M}$	2.43	50

enzyme, as demonstrated by inhibition with sulfhydryl reagents. The enzyme was treated with an —SH oxidizing agent, iodosobenzoate; an alkylating agent, iodoacetamide; and a mercaptide-forming agent, *p*-chloromercuribenzoate. Iodosobenzoic acid inhibited 15 per cent; iodoacetamide, 23 per cent; and *p*-chloromercuribenzoate, 89 per cent. In agreement with the low inhibiting effect of the oxidizing agent, iodosobenzoic acid, irradiation by x-rays had little effect on the enzyme activity. Irradiation with 1,000 r inhibited 13 per cent, while 2,000 r inhibited 18 per cent (Table IV).

3. *Adenosinetriphosphatase (Myosin)*.—While the enzyme activity of myosin is easily and accurately determined, under the conditions of these experiments extreme precautions had to be taken to secure reliable data. Rapid inactiva-

tion of the enzyme, when in dilute solutions and at high pH values, and the variation found in duplicate and triplicate experiments on the extent of inhibition produced by a given dose of x-rays had to be considered. For these reasons, when partial inhibition occurred, at least six samples were measured.

TABLE IV

Effect of Sulfhydryl Reagents and of X-Rays on the Activity of Yeast Crystalline Hexokinase

Enzyme activity measured at pH 7.9, veronal-acetate buffer. 12 micrograms of enzyme; 0.1 M glucose; 0.1 M adenosinetriphosphate. Temperature 20°. Time of incubation, 5 minutes. $E \left(\log \frac{I_0}{I} \right)$ proportional to 7 minutes hydrolyzable P which has been transferred to glucose in the reaction: glucose + ATP = glucose-6-phosphate + ADP.

System	E	Inhibition per cent
Enzyme—control.....	0.112	
“ + <i>p</i> -ClHg benzoate, 1×10^{-4} M.....	0.012	89.5
“ + iodoacetamide, 1×10^{-3} M.....	0.086	23.5
“ + iodosobenzoate, 1×10^{-3} M.....	0.095	15
“ + x-rays, 1000 r.....	0.098	12.5
“ + “ 2000 r.....	0.091	18.7

TABLE V

Effect of X-Rays on the Activity of Adenosinetriphosphatase (Myosin)

Nitrogen content of preparation used for irradiation, 0.02 mg. per cc. Buffer, bicarbonate-carbonate, 0.01 M, pH 9.1. The P figures give the amount formed in 10 minutes' incubation at 38°.

X-ray dose <i>r</i>	Phosphorus		Inhibition per cent
	Control micrograms	X-ray micrograms	
10	21.4	18.9	10
50	21.4	17.0	20
100	21.4	14.6	32
250	21.4	8.6	60
500	21.4	8.0	63
1000	21.4	1.1	95

When myosin was irradiated at pH 9.1 (bicarbonate-carbonate buffer), an inhibition was noticed after irradiation with 10 r and was complete with 1,000 r (Table V).

When myosin preparations are kept at 3° for a week (aged myosin) the enzyme activity is more sensitive to the inhibiting effect of mild oxidizing agents. The same increased sensitivity was observed towards x-rays. Aged myosin became so sensitive that 1 to 10 r produced marked inhibitions. Thus, 10

r and 8 r produced in excess of 50 per cent inhibition, while 1 r inhibited 30 per cent (Table VI).

Addition of glutathione produced a reactivation of the enzyme. Enzyme inhibition with 100 r was almost completely reversed on addition of glutathione. When the x-ray dose was increased to 500 r, reactivation by glutathione reached only 50 per cent (Table VII).

TABLE VI

Inhibition of Adenosinetriphosphatase (Aged Myosin) by Low Doses of X-Rays

Buffer, bicarbonate-carbonate, pH 9.1. Nitrogen content, 0.02 mg. per cc. Phosphorus liberated in 10 minutes at 38°.

X-ray dose	Phosphorus		Inhibition
	Control	X-ray	
r	micrograms	micrograms	per cent
1	11.9	8.4	30
5	11.9	5.4	55
10	11.9	5.7	52

TABLE VII

*Adenosinetriphosphatase Inhibition by X-Rays
Reactivation with Glutathione*

Buffer, bicarbonate-carbonate, pH 9.1. Nitrogen content of myosin, 0.02 mg. per cc. Glutathione (GSH), 0.02 M.

X-ray dose	Inhibition	Reactivation
	With x-ray	X-ray + GSH
r	per cent	per cent
10	10	80
100	22	64
500	41	56
1000	73	22

4. *Succinoxidase*.—Havard (24) reported that x-rays had slight effect on the activity of succinoxidase when irradiated with a dose of 20,000 r. These negative results were probably obtained because in the tissue suspensions the enzyme was not the rate-limiting step in the reaction. For the experiments on irradiation of succinoxidase, it was established first that 0.5 cc. of the enzyme suspension in phosphate buffer, pH 7.0, gave maximum O₂ uptake in the presence of 0.05 M succinate. With smaller amounts of enzyme the O₂ uptake was directly proportional to the enzyme concentration. Amounts of enzyme ranging from 0.4 cc. to 0.05 cc. were diluted to 1 cc. with phosphate buffer and were irradiated with 5,000 r. Inhibition was small when 0.2 to 0.4 cc. of the

enzyme suspension was taken. With 0.1 cc. of enzyme the inhibition rose to 29 per cent; with 0.05 cc. inhibition was complete (Table VIII). In the presence of glutathione, the inhibition dropped to 23 per cent. With glutamate (used by Dale (25) to protect *d*-amino acid oxidase from inhibition by x-rays), the inhibition was 62 per cent (Table VIII). X-ray inhibition was reversible.

TABLE VIII

Effect of X-Rays on the Activity of Succinoxidase

Buffer, phosphate, 0.02 M, pH 7.0; succinate, 0.05 M. Temperature 38°. Dose of x-rays, 5000 r. O₂ uptake in 50 minutes. Glutathione, 0.005 M.

Amount of enzyme <i>cc.</i>	O ₂ uptake		Inhibition <i>per cent</i>
	Control <i>c.mm.</i>	X-rays <i>c.mm.</i>	
0.4	290	270	7
0.3	235	214	9
0.2	147	132	10
0.1	82	58	29
0.05	25	0	Complete
0.05 + glutathione	34	26	23
0.05 + glutamate	26	10	62

TABLE IX

*Inhibition of Succinoxidase by X-Rays
Reactivation with Glutathione*

X-ray dose, 5000 r. Glutathione, 0.005 M added after irradiation.

Enzyme <i>cc.</i>	Inhibition	Reactivation
	With x-ray <i>per cent</i>	X-ray + GSE <i>per cent</i>
0.07	75	94
0.06	77	75
0.05	Complete	42

Addition of glutathione after irradiation reactivated the enzyme to varying degrees (Table IX).

Effect of X-Rays on Non-Sulphydryl Enzymes

For the study of the effect of x-rays on enzymes which do not require the presence of —SH groups for enzyme activity the following were used: trypsin, ribonuclease, and catalase, all of them in crystalline form, and lactic dehydrogenase and cytochrome oxidase.

1. *Trypsin*.—X-ray irradiation of impure trypsin (Fairchild's powdered

trypsin) was performed by Hussey and Thompson (26), who reported no effect from irradiation by large amounts of x-ray (2 hours in a 140 kv. machine, 4 ma. at a distance of 30 cm.).

Fifty micrograms of the enzyme was irradiated at two pH values, 7.5 and 9.1. There was no effect at all with 1,000 r, while 5,000 r inhibited 25 per cent (Table X).

TABLE X

Effect of X-Rays on the Activity of Crystalline Trypsin

Buffers, phosphate 0.05 M, pH 7.5 and bicarbonate-carbonate, 0.01 M, pH 9.1. Enzyme 50 micrograms per cc. E values ($\log \frac{I_0}{I}$) proportional to protein hydrolysis and enzyme activity.

X-ray dose <i>r</i>	pH	E		Inhibition <i>per cent</i>
		Control	X-ray	
1000	7.5	0.335	0.335	None
5000	7.5	0.335	0.255	24
1000	9.1	0.332	0.345	None
5000	9.1	0.332	0.245	26

TABLE XI

Effect of X-Rays on Crystalline Ribonuclease

Buffer, bicarbonate-carbonate, pH 9.1. Gas phase $N_2:CO_2$. Enzyme activity measured by CO_2 evolution on hydrolysis of ribonucleic acid.

Enzyme <i>micrograms</i>	X-ray dose <i>r</i>	CO_2 output		Inhibition <i>per cent</i>
		Control <i>c.mm.</i>	X-rays <i>c.mm.</i>	
25	1000	270	257	7
20	5000	256	189	26
10	5000	186	119	36
7.5	5000	163	92	44
5.0	5000	128	51	60

2. *Ribonuclease*.—This crystalline enzyme was irradiated at different concentrations. When 25 micrograms in 1 cc. was irradiated with 1,000 r and with 5,000 r there was no effect. When the amount of enzyme was progressively decreased while the amount of x-ray irradiation was kept constant, the inhibition increased steadily, from 26 to 60 per cent (Table XI). Undoubtedly this inhibition was due to protein denaturation, which usually occurs with high dose irradiation. Lea *et al.* (27) irradiated *dried* ribonuclease and found that irradiation of 2 mg. with 3.4×10^7 r destroyed 37 per cent of the activity of the enzyme.

3. *Lactic Dehydrogenase*.—The enzyme (163 micrograms in 1 cc.) was irradiated at two pH values, 7.2 and 9.1, and in two doses, 1,000 r and 5,000 r. An aliquot (0.07 cc.) was taken for measurement of activity. Enzyme inhibition was negligible (Table XII).

4. *Cytochrome Oxidase*.—Preliminary experiments showed that 0.05 cc. to 0.2 cc. of the cytochrome oxidase gave a linear relationship between O₂ uptake and enzyme concentration. For irradiation the original enzyme solution was

TABLE XII
Effect of X-Rays on Lactic Dehydrogenase

Buffers, 0.05 M phosphate, pH 7.5 and bicarbonate-carbonate, 0.01 M, pH 9.0. Enzyme, 163 micrograms per cc. for irradiations.

X-ray dose	pH	O ₂ uptake		Inhibition
		Control	X-ray	
r		c.mm.	c.mm.	per cent
1000	7.2	56.8	53.5	5.6
5000	7.2	56.8	53.0	6.5
1000	9.0	31.7	31.9	None
5000	9.0	31.7	30.3	4

TABLE XIII
Effect of X-Rays on Cytochrome Oxidase

Dose, 5000 r. Original enzyme diluted for irradiation.

Dilution of enzyme	O ₂ uptake in 10 min.	
	Control	X-rays
	c.mm.	c.mm.
1:5	173.1	166.3
1:10	103.2	102
1:15	45.0	43.0
1:20	25.5	25.7

diluted so as to contain the equivalents of 0.05, 0.1, 0.15, and 0.2 cc. of the original sample. The diluted samples were irradiated with 5,000 r at pH 7.0. No significant inhibition was obtained on irradiation with 5,000 r even at the lowest dilution used (Table XIII).

5. *Catalase*.—Tytell and Kersten (28) have reported that catalase (a non-sulfhydryl enzyme) is quite resistant to the action of x-rays, while urease (a sulfhydryl enzyme) was more easily inhibited. Four samples of crystalline catalase containing 9.0, 11.3, 15.0, and 22.5 micrograms of enzyme per cc. were irradiated with 5,000 r. 0.5 cc. of these irradiated samples was taken for the determination of enzyme activity. No inhibition was detected even at the highest dilution (Table XIV).

6. *Ionic Yields.*—It is customary in radiation chemistry to express the efficiency of a radiation in initiating chemical changes as the ratio of the number of molecules (M) which are decomposed to the number of ion pairs (N) produced in the system. The ratio $\frac{M}{N}$ is the ionic yield of the reaction. In gas reactions the most striking feature is the large number of molecules which can sometimes be brought into reaction compared with the number of molecules ionized.

TABLE XIV
Effect of X-Rays on Catalase

Dose, 5000 r. H_2O_2 , 0.005 N. Figures give cubic centimeters of H_2O_2 destroyed by 5 cc. of enzyme solution in 5 minutes.

Enzyme	H_2O	
	Control	X-rays
<i>micrograms</i>	<i>cc.</i>	<i>cc.</i>
9.0	1.72	1.97
11.3	1.20	1.14
15.0	1.08	0.98
22.5	0.69	0.70

TABLE XV
Ionic Yields of Enzymes Inhibited by X-Rays

Enzyme	Molecular weight	Ionic yield
Phosphoglyceraldehyde dehydrogenase.....	70,000	0.93
Trypsin.....	36,500	0.025
Ribonuclease.....	13,000	0.03
Carboxypeptidase*.....	35,000	0.16
<i>d</i> -Amino acid oxidase†.....	70,000	0.1

* From the manuscript of Dale, Meredith, and Gray quoted previously.

† From Lea's calculation (29).

These reactions with high ionic yields belong in most cases to the class of chain reactions. The ionic yield for carboxypeptidase inhibited by x-ray irradiation, as calculated by Dale, Meredith, and Gray,² is 0.16; that of *d*-amino acid oxidase, 0.1.

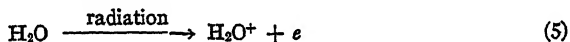
For the calculation of the ionic yields in Table XV the values of 1.616×10^{12} ion pairs liberated in 1 cc. of water per 1 r, and of 6.023×10^{23} for Avogadro's number, have been taken. There are, as yet, no reliable data for the molecular weight of phosphoglyceraldehyde dehydrogenase; it has been assumed to be 70,000. Since 70 micrograms per cc. of solution was irradiated, there would be

² Dale, W. M., Meredith, W. J., and Gray, L. H., The inactivation of an enzyme (carboxypeptidase) by x- and α radiation. Manuscript kindly sent to one of us by Dr. Gray.

6.023×10^{14} molecules of enzyme. Irradiation with 200 r (3.23×10^{14} ion pairs) produced half-inhibition, *i.e.* "destruction" of 3.01×10^{14} molecules, which gives an ionic yield of 0.93. Unfortunately, recent studies favor the opinion that adenosinetriphosphatase is a small protein adsorbed in myosin; hence, no ionic yields can be calculated from our data. The ionic yields of two non-sulphydryl enzymes, trypsin and ribonuclease, are 0.025 and 0.03 respectively. The ionic yield for ribonuclease is in remarkable agreement with that given by Lea (29) from unpublished data of Lea and Holmes (ionic yield, 0.03).

DISCUSSION

The experiments presented here have shown that when dilute aqueous solutions of enzymes are irradiated with x-rays, the sulphydryl enzymes, *i.e.* those requiring —SH groups in the protein moiety for enzymatic activity, are more susceptible to inhibition than enzymes which need no —SH groups for activity. It is postulated that inhibition of sulphydryl enzymes was produced by oxidation of the sulphydryl groups. The mechanism of this oxidation can best be explained by following Weiss's suggestion (30) of the series of reactions which might occur when water is irradiated with x-rays. The products first formed are the positive ion H_2O^+ and an electron:



Because of the high energy of hydration of H^+ , the reaction



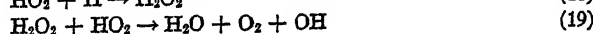
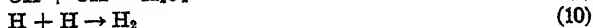
is highly exothermic and probably occurs soon after ionization. There is no likelihood of its persisting long enough to combine with the electron to form water again. The electron which was set free at the ionization will react with water:



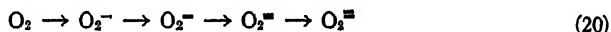
Decomposition of the H_2O^- ion by the exothermic reaction will give:



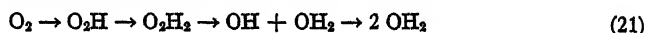
Furthermore, the following reactions are possible:



When oxygen is present in water there may occur a reduction of the molecule which, if it happens by univalent steps, according to Michaelis (31) there would be formation of



In water, where protons can be added, these would be converted to



Reduction of molecular oxygen would thus produce three powerful oxidizing agents, the radicals OH and O_2H , and H_2O_2 .

There are four powerful oxidizing agents among these series of reactions: the free radicals OH and O_2H , atomic oxygen, and H_2O_2 . Oxidation of the sulfhydryl groups of the protein by these agents will take place as indicated in Equations 1 to 4. Furthermore, the H_2O_2 produced during this oxidation will itself act as an oxidizing agent, thus increasing the yield of the reaction. Evidence for the occurrence of these oxidations was provided by enzyme reactivation on addition of glutathione, glutathione converting the disulfide groups back into the sulfhydryl stage. However, complete reactivation was possible only when small amounts of radiation were used and when inhibition was partial. As the dose of x-rays increased and inhibition became more complete, the degree of reactivation diminished. For example, irradiation of phosphoglyceraldehyde dehydrogenase with 500 r produced an inhibition of 50 per cent, and reactivation of 62 per cent on addition of glutathione. With this amount of irradiation, therefore, 62 per cent of the total inhibition was produced by oxidation of the sulfhydryl groups (reversible inhibition) and 38 per cent must have been produced by denaturation of the protein through rupture of the hydrogen bonds or oxidation of other oxidizable groups of the protein (irreversible inhibition). These two processes are different and are evidence that ionizing radiations in small amounts act specifically on the sulfhydryl enzymes by oxidation of the —SH groups *without destruction* of the protein molecule, while large amounts produce non-specific, protein denaturation.

Proteins possess two kinds of —SH groups: (1) freely reacting —SH groups, easily oxidized by mild oxidizing agents; and (2) *sluggish* —SH groups which are not oxidized by these agents, but react with mercaptide-forming agents (Hg, As, Cd, etc.). The sulfhydryl groups of hexokinase seem to contain these two kinds of —SH groups. The freely reacting —SH groups of hexokinase seem to be only about 15 per cent of the total —SH groups, as calculated from the inhibition with iodosobenzoate (mild oxidizing agent). Inhibition of the enzyme with x-rays (1,000 to 2,000 r) was only 12 to 18 per cent. In spite of the high oxidizing power of the OH and O_2H radicals, x-rays in moderate doses did not oxidize those sluggish —SH groups which could not be oxidized by iodosobenzoate.

The effect of very low doses of x-rays on aged preparations of adenosine-tri-phosphatase is quite remarkable since inhibition of enzyme activity was observed even after irradiation with 1 r. Such inhibitions are further proof that ionization of water by "one ion pair" starts a chain reaction and that several of the radicals produced react with the sulfhydryl groups of the enzyme. They also speak against the postulated short life of the radicals formed on ionization of water.

Inhibition of non-sulfhydryl enzymes by x-rays required a greater amount of x-ray irradiation per molecule of enzyme. Whether these inhibitions are due simply to the denaturation of the protein through rupture of hydrogen bonds by the products of the irradiation of water, or are due to chemical action on certain side chains of the molecule necessary for enzyme activity, cannot be answered.

SUMMARY

Dilute solutions of sulfhydryl enzymes (phosphoglyceraldehyde dehydrogenase, adenosinetriphosphatase, succinoxidase) showed reduced activity on irradiation by small amounts of x-rays. When the inhibition was partial the enzyme was reactivated on addition of glutathione. When the inhibition was more complete, reactivation was only partial. These observations are interpreted as being due to oxidation of the —SH groups of the protein by the products of water irradiation, the radicals OH and O_2H , and H_2O_2 and atomic oxygen. The irreversible inhibition which occurs when the dose of x-rays is increased is attributed to protein denaturation.

Inhibition of the non-sulfhydryl enzymes trypsin, catalase, and ribonuclease, which required larger amounts of x-rays, is attributed to protein denaturation.

These experiments are further evidence that inhibition of enzymes by ionizing radiations is due to the indirect action of the products of irradiated water rather than to direct ionization of the enzyme through collision with the ionizing radiation.

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MOVEMENTS OF WATER IN CELLS OF NITELLA

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The behavior of water in the living cell is highly important but our knowledge regarding it is very unsatisfactory. Since cells of *Nitella* are very favorable for such study some experiments have been made some of which are reported here.

They deal with the forces which cause water to enter and to leave the cell and show how such movements of water can be quantitatively predicted.

Since the cells show little or no permeability to sucrose the experiments have been made with solutions of sucrose in which the cell behaves as a fairly good osmometer.

Movements of water can be induced in cells of *Nitella* by placing water at one end, A, and a solution of sucrose at the other, B. Water then enters at A and passes to B where it escapes. Solutes in the cell move with the water from A to B but do not escape at B since they are unable to pass out through the protoplasm except very slowly. As a result the internal osmotic pressure decreases at A and increases at B.

The movement of water is due to the osmotic drive which forces water into the cell; this may be defined as the difference between the internal and the external osmotic pressure.

If the osmotic drive at A is greater than at B water moves from A to B. The osmotic drive falls off at A and increases at B until the two values become equal. The motion then stops.

Experiments have been made to determine the final equilibria attained and an equation has been obtained which enables us to predict the results. The agreement between prediction and observation is fairly satisfactory.

EXPERIMENTAL

The *Nitella* cell has a layer of protoplasm not over 15 microns in thickness surrounding a large central vacuole filled with sap (this is over 450 microns in diameter). Outside this is a cellulose wall about 15 microns thick.¹

¹ The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87) at 15°C. \pm 1°C. About 15 hours before use the cells were placed in a large amount of Solution A

The movement of water can easily be observed under the microscope if the *Nitella* cell² is placed on a slide with a barrier of vaseline in the center to prevent the water at A from mixing with the solution at B. The rush of water from A to B is easily visible because it carries with it particles suspended in the cell sap.

If the sap is stained with brilliant cresyl blue³ we see that the movement of water carries the dye with it so that the color becomes paler at A and deeper at B.

For quantitative work we may employ the method described below.

Cells 5 to 8 cm. long were placed in the apparatus shown in Fig. 1. A cork 1.7 cm. long and 8 mm. in diameter was split lengthwise by a sharp razor and a shallow groove was made in one half. A *Nitella* cell was placed in this surrounded by vaseline to make a water-tight seal and the whole of the flat surface of the cork was covered with vaseline. A glass tube R completely filled with water was then fitted over the right end and at the left end a tube L filled with water was fitted on; this tube ended in a calibrated capillary with scale divisions etched in the glass (the capillary was 15 cm. long). Care was taken to exclude air bubbles from both tubes.⁴

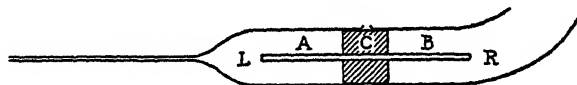


FIG. 1. Apparatus for measuring the movement of water in cells of *Nitella*. The cell is held under gentle pressure in the cork, C. The tubes L and R are filled with water and the apparatus is left until the meniscus in the capillary of L becomes stationary. Water in R is then replaced by a solution of sucrose. We then find that water enters the cell at A, passes along inside the cell, and escapes at B.

The diameter of the cork was such that the tubes compressed the cork enough to make a water-tight seal without injuring the cell. The seal was tested by tipping the apparatus to make sure that no movement of water occurred under the influence of gravitation. In use the apparatus was kept horizontal and was supported on a strip of wood in a groove of the right size to hold it firmly.

If the apparatus has been set up properly the meniscus at the end of the capillary will be stationary and not easily affected by adding or subtracting liquid at R.

At the start L and R were filled with water and the meniscus at the end of L was

in a room at about 25°C. and the temperature of the solution rose gradually to about 25°C. and the experiments were performed at about this temperature.

Microscopic observations show that the dimensions of the cell do not change during the experiments so that we may conclude that when a given amount of water enters at one end the same amount escapes at the other end.

The use of metal forceps was avoided.

² The movement of particles in the sap of the vacuole may occur without disturbing the normal protoplasmic movement (cyclosis). Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1947, **30**, 439.

³ Irwin, M., *J. Gen. Physiol.*, 1926, **9**, 561; 1926, **10**, 75.

⁴ If necessary the fitting of the tubes to the cork may be done with the cork submerged in water or in solution.

observed to see when it became steady. If this took longer than 10 minutes a new cell was introduced into the apparatus. When the meniscus had become steady the apparatus was tipped to make sure that there was a good seal at the cork in which case the meniscus did not move; if the seal was defective this was corrected.

The water in R was then removed by means of a pipette with as little mechanical disturbance as possible and a new solution was introduced into R.

The movement of liquid in the capillary was observed under a magnifying glass and the time was read on a stop watch (if necessary one observer watched the capillary and another the stop watch).

Microscopic observations of cells with sap containing dye showed that with water at A and sucrose solution at B any backward diffusion from B to A was negligible.

After each experiment the cells were kept under observation for 2 days. Unless they remained in good condition the experiment was rejected. Further observation does not appear to be necessary since if no signs of injury appear in 2 days they usually live indefinitely. The condition of the cell was judged by its turgor and its microscopic appearance.

All osmotic pressures are recorded at 25°C.

Equilibrium Values

If we know the internal and external osmotic pressure and the volumes of A and B we can predict the total amount of flow and the osmotic pressures at equilibrium when the osmotic drive at A (or D_A) becomes equal to that at B (or D_B). This final value when $D_A = D_B$ may be called D_F .

To illustrate the calculation of D_F we may consider a case where the combined lengths of A and of the cork amount to 5 cm. and they are both regarded as A because they act alike in giving up solute to B. We assume for convenience in calculation that the volume of A (or V_A) is 5 and that of B (or V_B) is 1.

We assume that the osmotic pressure in the cell is due to a single solute, S, and that in each section 1 cm. in length there are⁵ 6.4 α mols of S giving an osmotic pressure of 6.4 atmospheres (all values relate to 25°C.).⁶ We assume that S cannot pass out of the protoplasm.

If we put water at A and a solution of sucrose with an osmotic pressure of 6 atmospheres at B we have the following situation:

At A	At B
$P_{IA} = 6.4$	$P_{IB} = 6.4$
$P_{OA} = 0$	$P_{OB} = 6$
$D_A = 6.4 - 0 = 6.4$	$D_B = 6.4 - 6 = 0.4$

Here P_I is the internal and P_O the external osmotic pressure and D the osmotic drive. Since D_A is greater than D_B water enters at A, moves along inside the

⁵ The value of α might be of the order of 10^{-7} . It is assumed that the value of α is constant for each cell but that it may vary from cell to cell.

⁶ The value 6.4 was arrived at by plasmolytic experiments.

cell, and escapes at B. Since S is unable to pass out through the protoplasm it cannot escape from the cell and its concentration increases at B while it diminishes at A. Hence D_A becomes less and D_B increases until they become equal so that $D_A = D_B = D_F$.

Let us now consider the excess of osmotic drive at A as compared with B. If we remove this excess the osmotic drive will become equal throughout the cell. The excess is $D_A - D_B = 6.4 - 0.4 = 6$. To remove this we must take $6x$ mols of S from each of the 5 sections of A. Hence the amount removed is $V_A(D_A - D_B) = 5(6.4 - 0.4) = 30x$ mols of S. The osmotic drive is now 0.4 everywhere and if we distribute the $30x$ mols uniformly throughout the cell the osmotic drive will remain equal everywhere. For this purpose we divide the $30x$ mols into 6 equal parts and give 1 part to each section. Since the volume of the cell, or V_{cell} , is 6 we may write for the amount each section will receive:

$$\frac{V_A(D_A - D_B)}{V_{cell}} = \frac{5(6.4 - 0.4)}{6} = 5x \text{ mols}$$

When this is added to D_B we have:

$$\frac{V_A(D_A - D_B)}{V_{cell}} + D_B = \frac{5(6.4 - 0.4)}{6} + 0.4 = 5.4$$

This is now the value of the osmotic drive at all points and hence it is the value of D_F . We therefore have:

At A	At B
$P_{IA} = 6.4 - 6 + 5 = 5.4$	$P_{IB} = 6.4 + 5 = 11.4$
$P_{OA} = 0$	$P_{OB} = 6$
$D_A = 5.4 - 0 = 5.4$	$D_B = 11.4 - 6 = 5.4$

Since $D_A = D_B$ the motion stops. The value of D_F is 5.4.

The agreement between calculation and observation was tested in the following manner. Cells were selected in which the diameter of the vacuole (in which the water chiefly moves) was approximately the same as the bore of the capillary (473 microns). If the length⁷ of A is 3 cm. and the motion in the capillary from left to right is 2 cm. it is evident that 66.7 per cent of the liquid in A has moved to B. The cells in which the diameter of the vacuole was approximately equal to the bore of the capillary consisted of 2 lots. In the first lot of 4 cells the ratio $V_A \div V_{cell}$ in each cell was equal to 0.604. Water was placed at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B. The value of D_B was -4.8 hence that of D_F was 1.96 and the calculated loss at A was $100(6.4 - D_F) \div 6.4$ or $100(6.4 - 1.96) \div 6.4 = 69.4$ per cent. The average observed value as shown by the flow was 66.9 per cent.

In another lot of 7 such cells with a ratio of $V_A \div V_{cell}$ of approximately 0.548

⁷ This includes the area under the cork since this acts like A in giving up solute to B.

in each cell water was placed at A and 0.2 M sucrose with an osmotic pressure of 5.1 atmospheres at B. The average calculated value of the loss at A was 36.0 per cent and the observed value 35.3 per cent (Standard deviation 7.2).

Calculations of D_F show that the total volume of flow required to produce equilibrium if expressed as per cent of the volume of the cell is at a maximum when $V_A \div V_B = 1$ and falls off regularly as the value of this ratio increases or diminishes. This agrees in general with observation but there is considerable variability.

These observations show that the average amount of flow needed to produce equilibrium can be predicted with considerable accuracy. In making the calculations we assume that the movement of solute corresponds to the movement of liquid so that when half of the liquid in A moves to B half of the solute also moves. If not all the solute were equally affected by the flow the movement would exceed the predicted amount. But if the flow were stopped by the aggregation of colloidal masses in the sap before true equilibrium occurred the amount would be less than the calculated value.

The kinetics of flow present some interesting features which may be taken up in a later paper.

I wish to thank Mr. Jerome S. Fass for the care and skill he has shown in carrying out these experiments.

SUMMARY

When one end of a *Nitella* cell (A) is bathed in water and a solution of sucrose is placed at the other (B) we find that water enters at A, travels along inside the cell, and escapes at B. The solutes which cannot pass out through the protoplasm at B remain behind so that the osmotic pressure increases at B and diminishes at A until equilibrium is reached and the motion stops.

An equation is given which enables us to predict with considerable accuracy the amount of flow required to produce equilibrium.

TRANSPORT OF WATER FROM CONCENTRATED TO DILUTE SOLUTIONS IN CELLS OF NITELLA

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The transport of water from concentrated to dilute solutions which occurs in the kidney and in a variety of living cells presents a problem of fundamental importance.

If the cell acts as an osmometer we may expect to bring about such transport by creating an inwardly directed osmotic drive which is higher in one part of the cell than in other regions of the same cell. The osmotic drive is defined as the difference between internal and external osmotic pressure.

Experiments with *Nitella* show that this expectation is justified. If water is placed at one end of the cell (A) and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at the other end (B) water enters at A, passes along inside the cell, and escapes at B leaving behind at B the solutes which cannot pass out through the protoplasm. Hence the internal osmotic pressure becomes much higher at B than at A. When 0.4 M sucrose at B is replaced by 0.3 M sucrose with an osmotic pressure of 8.1 atmospheres we find that water enters at B, passes along inside the cell, and escapes at A so that water is transported from a concentrated to a dilute solution although the difference in osmotic pressure of the 2 solutions is more than 8 atmospheres. The solution at B thus becomes still more concentrated.

EXPERIMENTS

Cells of *Nitella*¹ 5 to 8 cm. long were employed. In these cells the protoplasm forms a layer not over 15 microns thick surrounding a large central vacuole over 450 microns in diameter; outside the protoplasm is a cellulose wall about 15 microns thick.

The cells were placed in the apparatus described in a previous paper² (see Fig. 1). The center of the *Nitella* cell was held under gentle pressure in a piece of cork 1.7 cm. long so as to make a water-tight seal. At the right end (B) the cell was surrounded

¹ The observations were made on *Nitella flexilis*, Ag. The cells were freed from neighboring cells and observed at once or kept in the laboratory in Solution A (cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1933-34, 17, 87) at 15°C. \pm 1°C. About 15 hours before use the solution containing the cells was allowed to warm up slowly to about 25°C. and the experiments were performed at about this temperature. All osmotic pressures are taken at 25°C.

² Osterhout, W. J. V., *J. Gen. Physiol.*, 1949, 32, 553.

by liquid in the tube R; at the left end (A) it was surrounded by water in the tube, L, ending in a calibrated capillary so that any movement of water in the tube could be measured by observing the movement of the meniscus in the capillary.

All the precautions described in the previous paper² were observed.

When water was placed at A and 0.4 M sucrose at B, water entered at A, passed along inside the cell, and escaped at B. This motion of water is due to the osmotic drive which forces water into the cell at A.

The internal osmotic pressure as judged by plasmolytic experiments with sucrose is about 6.4 atmospheres at 25°C. (all osmotic pressures are given at this temperature). Hence when we place water at A the osmotic drive tending to force water into the cell is $6.4 - 0 = 6.4$ atmospheres. Since the same situation exists at B the forces are equal and opposite and there is no motion of water. When we place water at A and sucrose solution at B the osmotic drive is greater at A and water enters at A, passes along inside the cell, and escapes at B. The water carries solutes from A to B which are unable to escape at B because they cannot pass out through the protoplasm. Hence the concentration of solutes and consequently the osmotic drive

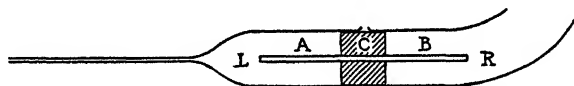


FIG. 1. Apparatus for measuring the movement of water in cells of *Nitella*. The cell is held under gentle pressure in the cork, C. The tubes L and R are filled with water and the apparatus is left until the meniscus in the capillary of L becomes stationary. Water in R is then replaced by a solution of sucrose. We then find that water enters the cell at A, passes along inside the cell, and escapes at B.

falls off at A and increases at B until the osmotic drive becomes equal at both places. The motion then stops.

Experiments made by placing water at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B gave 20 mm. as the average amount of flow in the capillary from A to B. The 0.4 M sucrose at B was then replaced by 0.3 M sucrose, with an osmotic pressure of 8.1 atmospheres. Water then entered at B, passed along inside the cell, and escaped at A (Fig. 2). The cell therefore transferred water from 0.3 M sucrose at B with an osmotic pressure of 8.1 atmospheres to water at A. The water thus transferred escaped into the water surrounding the cell at A.

This behavior of water can be demonstrated by placing the cell on a microscope slide with a barrier of vaseline in the center to keep the sucrose solution at B from mixing with the water at A. Observing the cell under the microscope we see that as water enters the cell at A and moves to B there is a rapid movement from A to B of particles suspended in the sap of the vacuole.³ If the sap is stained with brilliant cresyl blue³ the dye moves from A to B and becomes paler in color at A and deeper at B since it does not escape through the protoplasm.

When the 0.4 M sucrose at B is replaced by 0.3 M sucrose there is a rapid movement

³ The movement of particles in the sap of the vacuole may take place while the normal protoplasmic motion continues. Regarding the dye see Irwin, M., *J. Gen. Physiol.*, 1926, 9, 561; 1926, 10, 75.

of particles in the sap from B to A and if dye is present it becomes paler in color at B and deeper at A. The experiment may be varied by removing the water surrounding A and replacing it by mineral oil⁴ leaving only a film of water adhering to the cellulose wall. When water moves from B to A we see drops of water emerging into the oil at A.

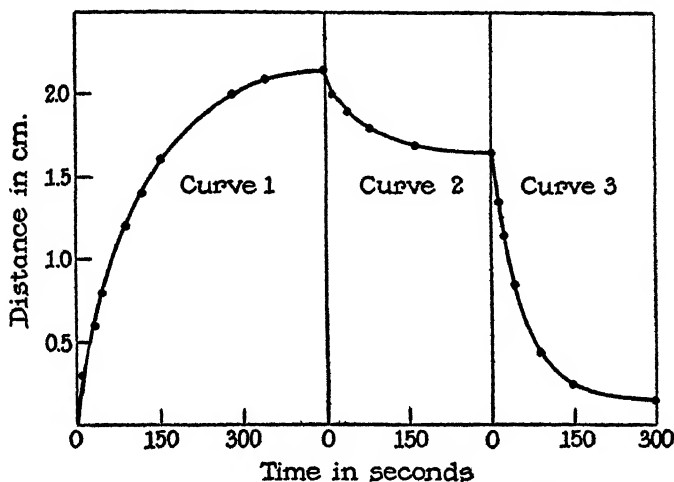


FIG. 2. Time curves of the flow of water in a typical experiment. Curve 1 shows the flow from A to B with water at A and 0.4 M sucrose at B.

Curve 2 shows the flow from B to A when 0.4 M sucrose at B is replaced by 0.3 M sucrose; this flow carries water from the external solution of 0.3 M sucrose with an osmotic pressure of 8.1 atmospheres at B to external water at A, thus increasing the concentration of the sucrose solution at B.

Curve 3 shows the flow from B to A when 0.3 M sucrose at B is replaced by water. This carries water from the external water at B to the external water at A.

In curves 2 and 3 the ordinates should be read downwards since the flow in the capillary is from right to left.

The length of A including the area under the cork (1.7 cm.) was 2.9 cm. and that of B was 1.8 cm.

When the 0.3 M sucrose at B was replaced by water there was a flow in the capillary from right to left and water escaped at A (Fig. 2). This was confirmed by microscopic observation.

Calculations

Microscopic measurements show no change in the dimensions of the cells during the experiments so that we may assume that when a given amount of water leaves the cell at A the same amount enters at B.

⁴Heavy mineral oil for medicinal use may be used. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1947, **30**, 439.

We may assume for convenience that each section of the cell having a length of 1 cm. contains $6.4 \times$ mols of a solute, S, giving an osmotic pressure of 6.4 atmospheres at 25°C . We assume that this is the only solute present and that it cannot pass out through the protoplasm.

When we place water at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B we have the following situation:

At A	At B
$P_{IA} = 6.4$	$P_{IB} = 6.4$
$P_{OA} = 0$	$P_{OB} = 11.2$
$D_A = 6.4 - 0 = 6.4$	$D_B = 6.4 - 11.2 = -4.8$

Here P_I is the internal and P_O the external osmotic pressure and D is the osmotic drive.

Since D_A is greater than D_B water enters at A, passes along inside the cell, and escapes at B leaving behind the solutes in the sap which are unable to pass out through the protoplasm. Hence the osmotic drive decreases at A and increases at B until both values become equal and the motion stops. The final value when $D_A = D_B$ may be called D_F . This may be calculated by means of the equation given in a former paper:²

$$D_F = \frac{V_A(D_A - D_B)}{V_{\text{cell}}} + D_B$$

Here V_A is the volume of A and V_{cell} the volume of the cell.

Let us consider a case where $V_A \div V_B = 1$. The area under the cork is regarded as part of A since it acts like A in giving up water to B. Since only relative volumes are required we may for convenience put $V_A = 1$ cm., $V_B = 1$ cm., and $V_{\text{cell}} = 2$ cm.

Substituting numerical values we have:

$$D_F = \frac{1(6.4 - (-4.8))}{2} + (-4.8)$$

$$= 0.8$$

Hence when the motion stops we have:

At A	At B
$P_{IA} = 6.4 - 5.6 = 0.8$	$P_{IB} = 6.4 + 5.6 = 12.0$
$P_{OA} = 0$	$P_{OB} = 11.2$
$D_A = 0.8 - 0 = 0.8$	$D_B = 12.0 - 11.2 = 0.8$

This means that from A with a length of 1 cm. $5.6 \times$ mols of S have moved over to B thereby lowering the osmotic pressure at A from 6.4 to $6.4 - 5.6 = 0.8$. When this is added to the $6.4 \times$ mols of S already present in B we have $12.0 \times$ mols in a section 1 cm. long and consequently the osmotic pressure is 12.0 atmospheres. The loss at A is $(5.6 \div 6.4) 100 = 87.5$ per cent.

If we now replace the 0.4 M sucrose at B by 0.3 M sucrose having an osmotic pressure of 8.1 atmospheres we have:

At A	At B
$P_{IA} = 0.8$	$P_{IB} = 12.0$
$P_{OA} = 0$	$P_{OB} = 8.1$
$D_A = 0.8 - 0 = 0.8$	$D_B = 3.9$

As D_B is greater than D_A water enters at B, passes along inside the cell, and escapes at A leaving behind the solutes which cannot pass out through the protoplasm. Hence the osmotic pressure falls off at B and increases at A until the motion stops. We then have $D_A = D_B = D_F$. We may calculate the value of D_F as follows. Since the motion is from B to A we write:⁵

$$\begin{aligned} D_F &= \frac{V_B(D_B - D_A)}{V_{\text{cell}}} + D_A \\ &= \frac{1(3.9 - 0.8)}{2} + 0.8 \\ &= 2.35 \end{aligned}$$

We then have:

At A	At B
$P_{IA} = 0.8 + 1.55 = 2.35$	$P_{IB} = 12.0 - 1.55 = 10.45$
$P_{OA} = 0$	$P_{OB} = 8.1$
$D_A = 2.35 - 0 = 2.35$	$D_B = 10.45 - 8.1 = 2.35$

This means that 1.55 x mols of S have moved from B to A raising its internal osmotic pressure from 0.8 to 2.35 atmospheres.

The loss at B is 100 $(1.55 \div 12.0) = 12.9$ per cent.

If the 0.3 M sucrose at B is replaced by water we have the following situation:

At A	At B
$P_{IA} = 2.35$	$P_{IB} = 10.45$
$P_{OA} = 0$	$P_{OB} = 0$
$D_A = 2.35 - 0 = 2.35$	$D_B = 10.45 - 0 = 10.45$

Since D_B is greater than D_A water moves from B to A. We may calculate the value of D_F as before:

$$\begin{aligned} D_F &= \frac{1(10.45 - 2.35)}{2} + 2.35 \\ &= 6.4 \end{aligned}$$

⁵ In the forward movement from A to B the area under the cork is regarded as part of A since it acts like A in giving up solute to B. In the backward movement from B to A the area under the cork is regarded as part of A since it acts like A in receiving solute from B.

We then have:

At A	At B
$P_{IA} = 2.35 + 4.05 = 6.4$	$P_{IB} = 10.45 - 4.05 = 6.4$
$P_{OA} = 0$	$P_{OB} = 0$
$D_A = 6.4 - 0 = 6.4$	$D_B = 6.4 - 0 = 6.4$

This means that $4.05 \times$ mols of S have moved from B to A raising its internal osmotic pressure from 2.35 to 6.4 atmospheres.

The fact that we arrive at the value 6.4 for D_F shows that the method of calculation is correct since this was the value at the start.

The loss at B is $(4.05 \div 10.45) 100 = 38.8$ per cent.

The total loss of S at B in 2 steps is $1.55 + 4.05 = 5.6 \times$ mols which is the amount moving from A to B.

The cell is now restored to its original state at the start of the experiments. It is in contact with water at A and B and has 6.4 atmospheres of pressure at all points.

The agreement between calculation and observation was tested by selecting cells in which the diameter of the vacuole (in which the liquid chiefly moves) was approximately the same as the bore of the capillary (473 microns). Then if A is 3 cm. long it is evident that a movement of 2 cm. in the capillary from A to B means a loss of 66.7 per cent at A. A lot of 4 cells was used in each of which the value⁶ of $V_A \div V_{cell}$ was 0.604. With water at A and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at B we have $D_A = 6.4$, $D_B = -4.8$, and $D_F = 1.96$. The calculated loss at A is $100 (6.4 - 1.96) \div 6.4 = 69.4$ per cent. The average observed loss was 66.9 per cent. Another lot containing 10 such cells in each of which the value of $V_A \div V_{cell}$ was approximately 0.599 gave for the average calculated loss at A 70.1 per cent and for the average observed loss 71.4 per cent.⁷

In the backward movement from B to A when 0.4 M sucrose is replaced by 0.3 M sucrose and then by water (Fig. 2) we might expect less flow than in the forward movement. Although the amount of solute which moves backward is the same as that which moves forward the concentration of the sap is higher in the backward movement. The backward flow is often less than the forward flow (Fig. 2) but as a rule it is greater than the predicted amount. This may be due to a greater dilution of the sap by the incoming water at B.

Returning to the hypothetical case where $V_A = 1$ cm. and $V_B = 1$ cm. we may say that if the forward flow is 87.5 per cent of 1 cm. or 0.875 cm. (page 562) we should expect the backward flow when 0.4 M sucrose is replaced by 0.3 M sucrose to be 0.129 cm. (page 563) and the backward flow when 0.3 M

⁶ Here V_A includes the area under the cork since it acts like A in giving up solute to B.

⁷ The standard deviation is 16.8.

sucrose is replaced by water to be 0.388 cm. (page 564) making a total of 0.517 cm. as compared with the forward flow of 0.875 cm.

We cannot expect close agreement in all cases between calculation and observation since there are variables which cannot be controlled. The calculation assumes a close correspondence between the movement of liquid and the movement of solute so that if half the liquid moves from A to B half the solute moves also. But if the entering water does not affect all of the solute equally the flow of water may be greater than expected. Or if the flow is stopped by the accumulation of colloidal masses in the vacuole so as to produce a stoppage before equilibrium is attained the flow will be less than expected.

DISCUSSION

The experiments show that water may move from a concentrated to a dilute solution when the osmotic drive is greater in one part of the cell than in other regions. Such a condition could doubtless arise if metabolism were not uniform throughout the cell and this would be favored if different regions of the cell were in contact with different external situations.

To maintain a flow of water from a concentrated solution at B to a dilute solution at A by metabolism it would be necessary to produce osmotically active substance inside the cell at B which would escape at A or become osmotically less active at A. Such a process might be periodic rather than continuous and during the periods when no transport from concentrated to dilute solutions occurred the solutes in the cell might alter so as to make such transport possible in a subsequent period.

If a substance M at B were converted to a substance N with a lower molecular weight the internal osmotic pressure at B would increase and water might move from B to A even if the external osmotic pressure were higher at B than at A. If N moved with the water inside the cell from B to A and there became polymerized and diffused back to B the process might repeat itself indefinitely, giving a periodic transfer of water from B to A.

It would be necessary to have some mechanical restraint to prevent indefinite expansion of the cell due to the incoming water. Such restraint is provided in plants by the cellulose wall and in animals by the mechanical properties of the tissue.

This mechanism may bring about the secretion of water as described in a previous paper.⁸

A thermodynamical treatment of the possible effectiveness of metabolism in causing movement of water has been given by Franck and Mayer.⁹ It would seem from this that a rather high degree of efficiency is possible.

⁸ Osterhout, W. J. V., *J. Gen. Physiol.*, 1947, **30**, 439.

⁹ Franck, J. and Mayer, J. E., *Arch. Biochem.*, 1947, **14**, 297.

I wish to thank Mr. Jerome S. Fass for the care and skill he has shown in carrying out these experiments.

SUMMARY

The transport of water from concentrated to dilute solutions which occurs in the kidney and in a variety of living cells presents a problem of fundamental importance.

If the cell acts as an osmometer we may expect to bring about such transport by creating an inwardly directed osmotic drive which is higher in one part of the cell than in other regions of the same cell. The osmotic drive is defined as the difference between internal and external osmotic pressure.

Experiments with *Nitella* show that this expectation is justified. If water is placed at one end of the cell (A) and 0.4 M sucrose with an osmotic pressure of 11.2 atmospheres at the other end (B) water enters at A, passes along inside the cell, and escapes at B leaving behind at B the solutes which cannot pass out through the protoplasm. Hence the internal osmotic pressure becomes much higher at B than at A. When 0.4 M sucrose at B is replaced by 0.3 M sucrose with an osmotic pressure of 8.1 atmospheres we find that water enters at B, passes along inside the cell, and escapes at A so that water is transported from a concentrated to a dilute solution although the difference in osmotic pressure of the 2 solutions is more than 8 atmospheres. The solution at B thus becomes more concentrated.

It is evident that if metabolism produces a higher osmotic pressure and consequently a higher inwardly directed osmotic drive in one region of the cell as compared with other parts of the same cell water may be transferred from a concentrated to a dilute solution so that the former solution becomes still more concentrated.

A SIMPLIFIED APPARATUS FOR PHOTOMETRIC ANALYSIS AND PHOTOMICROGRAPHY

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INTRODUCTION

A significant advance in the technique of histology and cytology is the current trend toward the use of quantitative methods of studying microscopical preparations; in other words, of substituting an objective measure of absorption for expressions like "more basophilic," less Feulgen-positive," etc. Among such methods are those which Caspersson has developed for determining the absorption spectrum of a small part of a cell, investigations which yield precise information as to the character and amount of a naturally occurring substance (Caspersson, 1936, 1947). This technique is elaborate, and requires so much knowledge of a special field of physics that the average histologist is unlikely to be trained for such work. Fortunately, a much simpler procedure is possible if one knows the nature of the absorbing substance to be estimated, as for example, in the determination of hemoglobin in an erythrocyte (Thorell, 1947), in analysis for protein in a nucleus (Pollister and Ris, 1947), or in measurement of the dye in a stained nucleus (Di Stefano, 1948). In such cases, if the substance is in sufficiently high concentration, one can proceed much as in an ordinary photometric chemical analysis and obtain information as to the amount of material in a small part of a cell, such as the nucleolus—even though this amount is of the order of 10^{-9} mg. Among the most abundant and physiologically important substances in cells are the nucleic acids and proteins; and these are the chief constituents responsible for staining an ordinary histological preparation. Indeed, it appears that with the common histological fixing methods a cell structure is mainly a nucleoprotein picture. Therefore, if one is to investigate the significance of cellular structure, an analysis for nucleic acids and proteins is probably the most valuable quantitative cytochemical study which can be undertaken. Recently Pollister and Ris (1947) have described briefly some procedures and an apparatus for nucleoprotein determination in cytological preparations. These are considerably simplified, and experience has shown that their use can be acquired readily by a worker who is trained in histology or cytology. Since then, the photometric apparatus, designed for use with the Photovolt photometer, has been much improved, especially in

the shielding of the light-sensitive element for satisfactory operation with photomultiplier tubes. The new device is adapted for photometric analysis at any visible wave length and for 2537Å in the ultraviolet spectrum. Since it is often useful to have a photographic record of a cell on which an absorption measurement has been made, the apparatus has been constructed with a built-in camera and with means of focussing both the visible and ultraviolet images for photomicrography. This one instrument, combined with the commercially available Photovolt photometer¹ and the appropriate microscopical equipment, is adequate for photomicrography and for photometric chemical analysis of substances within individual cells of a microscopical preparation. Examples of the application are obvious: the amounts of natural intracellular colored substances, such as pigments, can be estimated; cells may be stained and the amount of combined dye estimated; the intensity of color reactions, such as Millon's test, can be measured; and the natural ultraviolet absorption of such substances as nucleic acids and nucleotides can be determined. Combined with specific solubilities and specific enzyme action, the photometric method opens a wide field of quantitative chemistry at the cytological level (Pollister and Ris, 1947). The present note includes a general description of the apparatus, adequate construction details, and the essentials of the operating procedure.

General Description

The apparatus, in position for photometry, is shown in Fig. 1. It is mounted on the pillar of a heavy photomicrographic apparatus (Zeiss) in place of the original camera. There are two main parts to the apparatus, the *camera box* (C) and the *scanner* or photometric device (S). The former is a rigid, light-tight box (Figs. 1 and 2) connected to the microscope by a cloth sleeve. The projected microscopic image enters the camera through a circular opening in the bottom of the box, and passes to the photographic plate compartment (PC) through a 6 × 9 cm. opening, the plate aperture. In the lower back part of the camera is a mirror which is so mounted that when the front side of the camera is opened the operator can see, in the mirror, the image of the plate aperture. This part of the apparatus is for focussing the ultraviolet image on the lower surface of a fluorescent screen placed in the plate compartment. The plate compartment holds a 9 × 12 cm. metal plate holder, in such a position that its inner half is above the plate aperture. A first exposure is made on this part of the plate, and the plate is turned end for end in the plate holder for the second exposure. For focussing the visible image a clear glass plate the same size as the plate aperture is inserted in the plate compartment. On the lower surface of this plate are cross-lines which intersect at the center. For focussing the ultraviolet image there is inserted a plate the

¹ This apparatus is manufactured by Photovolt Corporation, New York. On special order search units with photomultiplier tubes are available.

lower surface of which is coated with artificial willemite, which fluoresces brightly in ultraviolet light, especially at wave length 2537\AA (Lavin and Pollister, 1942, and Lavin, 1943). Such screens are made to order by E. I. Dupont, Patterson Screen Division, Towanda, Pennsylvania. It is convenient to have the screen on a 9×12 cm. photographic plate from which the emulsion has been removed, for the screen may then be kept free of dust in a plate holder.

In the upper wall of the plate compartment, directly above the center of the plate aperture, is a circular opening through which the microscopic image is projected to the scanner. The scanner (figs. 2 and 3) consists of two plates, the lower (*LP*) and upper (*UP*), both of which can be moved horizontally, in a plane which is perpendicular to the axis of projection of the microscopic image. The movement of the lower plate is random within the horizontal plane and is limited by the size of the holes through which pass the two pillars (*PIL*). The lower plate can be locked in any position by tightening the nuts (*LPL*) above the large washers on the pillars. In the lower plate is an iris diaphragm (*D*), mounted so that when the lower plate is at the center of its range of movement the diaphragm is directly above the center of the plate compartment. The size of the aperture of the diaphragm can be read from the position of the diaphragm lever (*DL*) on the calibrated scale (*DS*). The upper plate (*UP*) of the scanner can be rotated horizontally through a short arc, the center of which is the shaft (*SH*). This uppermost plate carries the focuser (*F*) and the phototube search unit (*SU*) of the Photovolt photometer. The focuser consists of a mirror (*FM*) and telescope (*T*) which can be focussed on any level of the projection path, from the plate compartment to diaphragm.² There are two openings in the upper plate, one below the focuser (*FAP*) the other beneath the search unit (*SUAP*). The movement of the upper plate is limited by stops (*ST*) which can be adjusted so that when the plate is at one end of the arc of rotation the aperture to the focuser is directly above the center of the iris diaphragm of the lower plate, while at the opposite end of the arc the search unit aperture is in this position. The upper plate can be locked in either position by the nut (*UPL*).

Construction Details

Construction details are shown in Figs. 2 and 3. The materials are easily obtained, and the average laboratory shop contains all the tools needed. The apparatus must be strong, but at the same time as light as possible. The plate surfaces which are to slide over one another in the movements of the scanner should, of course, be perfectly flat and smooth, since they must move easily and also serve to exclude extraneous light from the interior of the apparatus. Satisfactory materials for these sliding plates (*i.e.* upper wall of the plate com-

² A simple focussing magnifier such as is regularly furnished with photomicrographic outfits may be used instead of the mirror and telescope.

partment, the upper and lower surfaces of the lower plate, and the upper plate) are masonite or duralumin. Since the weight of the whole apparatus is carried on the pillar by clamps (*CP*, Fig. 1) attached to the back of the camera, this wall must be of heavy plywood or hardwood. The door at the front of the camera is thin aluminum. The side walls of the camera and the non-sliding parts of the plate compartment and lower part of the scanner are of light plywood. The holes in the lower plate of the scanner through which pass the pillars (*PIL*) should be at least as large as the maximum aperture of the iris diaphragm of the scanner; the locations of the holes and the size of the washers should be chosen to allow full swing of the upper scanner plate at any position of the lower scanner plate. The smaller fittings may be made of brass.

Operation

(a) *Photomicrography with Visible Light*.—First, the object to be photographed is moved to the center of the microscopic field. Next the camera scanner is swung into position above the microscope, and the sleeve is arranged to make a light-tight connection with the microscope. Into the plate compartment is inserted the clear glass plate. Next, the scanner diaphragm is opened, and the scanner is moved about until the point of intersection of the cross-lines on the glass plate is visible in the focuser. The telescope is adjusted to bring the cross-lines into sharp focus. The projected microscopic image is then focussed sharply, the glass plate is removed, a loaded plate-holder is inserted, and the photographic exposure is made in the usual manner.

(b) *Ultraviolet Photomicrography at Wave Length 2537Å*.—The following procedure is satisfactory when one is using the Zeiss ultraviolet equipment, which consists of quartz monochromatic objectives, quartz eyepieces, and condenser with interchangeable top lenses for different numerical apertures. Except for the light source and the absence of a collector lens, our equipment is identical with that figured and described by Köhler (1904).³ The light is a Hanovia Sc2537 mercury arc, an important characteristic of which is that approximately 85 per cent of the light output is in the spectral region of wave length 2537Å. The lamp is a 24 inch quartz tube, with offset electrodes, and a polished observation window in front of the end of the horizontal part of the U-shaped arc. A circular slit (diameter about 2.0 mm.) is mounted in front of this window, a biconvex quartz lens collimates the light, it is then passed through two 60° quartz prisms of the Zeiss monochromatic illuminator, and from the second prism emerges a series of images of the slit. The dispersion of the instrument is such that the 2537Å image is several centimeters away from the 4358Å visible blue image (at 10 cm. from the face of the prism). The only other ultraviolet image which is bright enough to be visible on a fluorescent screen is a very faint 3650Å one, which is some distance away from the 2537Å spot. The monochromatic illuminator is placed far enough away from the quartz prism below the microscope so that the 2537Å image of the slit fills the entire aperture of the condenser. (As Lavin and Pollister, 1942, have pointed out, one can also get approximately monochromatic 2537Å light from this

³ Similar quartz equipment is now being manufactured by Cooke, Troughton, and Sims, Ltd., New York representative E. A. Schrag, New York.

type of lamp by use of a liquid filter of cobalt and nickel sulfates (Bäckstrom, 1940), without any prism monochromator.)

In the procedure for ultraviolet photomicrography, the image is first oriented at the center of the glass plate with approximately monochromatic visible light (for example, the green light isolated from a tungsten lamp by a Wratten 62 filter) as described above for visible photomicrography. The glass plate is then replaced by the fluorescent screen. The ultraviolet image is brought into approximate focus by the proper number of turns of the fine adjustment (e.g. with a 2.5 mm. quartz monochromatic objective one must focus downward five and one-quarter complete revolutions of the fine adjustment in order to go from the green to the 2537Å image). The condenser diaphragm is next opened wide, the ultraviolet light is admitted to the microscope, and the room is darkened. The front of the camera is opened and the fluorescent screen can then be seen in the mirror within the camera. The objects which absorb considerable ultraviolet light, such as cell nuclei, will appear dark on the screen, other parts lighter. When using lower magnifications these dark areas can at once be brought into sharp focus, but for higher magnifications this is most successful only after one has become well dark-adapted (20 to 30 minutes). For this waiting period it is useful to have dark-adaptation red goggles, such as those in the Polaroid kit No. 1084. It becomes possible, with practice, to obtain sharp focus for every 2537Å photograph. Once the focus on the screen is satisfactory the photomicrograph is made in the usual manner.⁴

(c) *Photometry with Visible Light.*—Before undertaking the first routine measurements of transmission the apparatus must be properly aligned. The lamp and the optical elements of the microscope should be carefully centered in the usual manner. The microscope should be placed so that when the scanner diaphragm is in the center of its range of movement the margin of the diaphragm opening is concentric with the microscopic field. The phototube is aligned in the following way. The condenser is focussed sharply, using the particular spectral region which is to be employed in the absorption measurements. Next, an object is centered in the opened scanner diaphragm, the telescope is focussed sharply on the edge of the scanner diaphragm, and the object sharply focussed at this level. Then, with the mechanical stage, the slide is moved to bring an empty region, outside the tissue, into the field. By rotation of the upper plate the phototube search unit is then moved into position above the diaphragm, and locked in place. The condenser iris diaphragm is closed until the galvanometer reading is approximately 50. The clamps holding the search unit in place are loosened, and the unit moved about until the position which gives maximum galvanometer deflection has been found. The search unit is clamped in this roughly centered position, and the scanner diaphragm slowly closed until the galvanometer reading begins to fall. Leaving the diaphragm set at this aperture the search unit is again moved about to obtain a maximum reading, and the scanner diaphragm aperture at which the reading decreases is again found. If necessary the above procedure should be repeated several times, until the phototube is centered above the diaphragm and the scanner diaphragm opening which admits a light spot covering the whole width of photosensitive surface of the phototube has been found.

⁴ Useful types of plates for 2537Å photomicrography are Eastman Spectroscopic III-O, Wratten Metallographic, and Eastman Process. Orthochromatic and panchromatic emulsions are unsatisfactory.

It is well at this time to check the uniformity of illumination of the microscopic field, by making a series of galvanometer readings at scanner diaphragm apertures below that which gives maximum reading. These values should be directly proportional to the area of the diaphragm aperture. If this is not so, the field should be carefully checked for uniformity of illumination, either by visual examination, or by a photograph, or by exploration using a small scanner diaphragm aperture. Improper alignment of the optical elements is another possible source of non-linearity. If the failure of direct proportionality is noted only with the larger diaphragm openings it is likely that an area greater than the photosensitive area is being illuminated, and readings must be made with the smaller apertures where the linear relationship holds. The maximum usable scanner diaphragm aperture is an important datum for the apparatus. Where possible the magnification and the projection distance should be selected so that one uses at least three-fourths of the photosensitive surface.

The routine photometry is carried out in the following manner. The object is centered in the scanner diaphragm with the mechanical stage, focussed sharply with the telescope, and the diaphragm closed to circumscribe an area one wishes to measure. The light to the microscope is cut off, and the search unit locked in recording position. The galvanometer is adjusted to zero, and the light admitted to the microscope. After a slight movement of the fine adjustment to obtain the lowest galvanometer deflection (which indicates that the image is in focus on the photosensitive surface) the galvanometer deflection is recorded. This *first reading* measures the intensity of light after it has passed through the specimen. Next, the light is cut off, the slide is moved to a stage vernier reading known to be an empty area outside the section, and a reading made through this region. This *second reading* measures the light transmitted through the optical system when no specimen is in the field, and for purposes of calculation this is an intensity of 1.0. The first reading divided by the second is the *transmission* (T) of the specimen. The logarithm of the reciprocal ($\log_{10} 1/T$) is the *extinction* (E) of the specimen. Extinction is a value which varies directly with concentration and thickness of specifically absorbing substance according to the Beer-Lambert law, which can be expressed in simplified form as $E = kcd$ where k is a constant, c is concentration, and d is thickness of absorbing layer (see Brode, 1943, and Gibb, 1942). Further details of computations and corrections for photometry of microscopical preparations are discussed by Caspersson (1936), Thorell (1947), Pollister and Ris (1947), and Di Stefano (1948).

(d) *Photometry with Ultraviolet Light of Wave Length 2537 Å.*—This wave length is near the absorption maximum of the heterocyclic bases of the nucleotides. The ultraviolet photometric procedure is only slightly more complicated than that just described; the image is centered with visible light, and the whole transmission is carried out without any necessity for observing the ultraviolet image on a fluorescent screen. With the quartz monochromatic objectives of Köhler the ultraviolet image is always somewhat larger than a visible image projected the same distance. One can be sure therefore, that if an area is circumscribed by the scanner diaphragm in visible light, a somewhat smaller central part (80 to 90 per cent) of this same area will be projected into the phototube by ultraviolet light.⁵

⁵ Because of the larger size of the ultraviolet image it is important to orient the area to be measured so that its center coincides with the center of the microscopic

The following is the photometric routine. The monochromatic (e.g. green) visible image is focussed and centered in the scanner diaphragm, and the latter is closed to circumscribe the desired area. The visible light is cut off, leaving the shutter⁶ to the ultraviolet light closed. The phototube search unit is locked in recording position, and the fine adjustment rotated to bring the ultraviolet image into approximate focus. The shutter is opened to admit the ultraviolet light, and by slight movement of the fine adjustment the position of lowest reading is found, indicating that the 2537Å image is focussed on the photosensitive surface. The readings for specimen and vacant area are then made as described above for visible photometry. To continue with a second transmission measurement the fine adjustment is changed to the visible focus, and the section then brought back into the field.

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field. If the object is excentric the change of size of the field area with the change of focus from the visible to the ultraviolet image causes the image of the object to move laterally, and hence partially out of the field of transmission measurement.

⁶ A simple shutter (SR, Fig. 1), in the form of a metal plate, is mounted on a shaft at one side of the foot of the microscope. The shutter can be rotated horizontally beneath the microscope condenser to cut off the ultraviolet light. On the upper surface of the shutter plate is a microscope mirror, which when the shutter is in closed position serves to reflect visible light into the microscope. Another simple shutter and a filter holder are mounted in front of the tungsten lamp.

DESCRIPTION OF FIGURES

FIG. 1. The photometric-photomicrographic apparatus in position for photometry with the ultraviolet microscope. The front of the camera is closed.

FIG. 2. Diagram of the assembled apparatus, with the scanner unit raised some distance above the camera and the front of the camera opened for focussing the ultraviolet image.

FIG. 3. Assembly diagram of the scanner unit. The scale is shown in the lower left corner. The sides have not been foreshortened, and measurements may be taken directly from vertical, front, and side surfaces for projection of plane blueprints or templates.

Abbreviations

C, camera.

C AP, camera aperture.

CL, clamps, holding search unit in position.

CP, clamp holding camera to pillar of photomicrographic apparatus.

D, iris diaphragm.

DL, diaphragm lever.

DS, diaphragm scale.

F, focuser, for photomicrography and for centering image for photometry.

F AP, focuser aperture, an opening in the upper plate through which the microscopic image is projected to the focuser.

FM, mirror of focuser.

L, tungsten lamp.

LB, box enclosing ultraviolet lamp and monochromator.

LP, lower plate of scanner.

LPL, lower plate lock, consisting of washers and nut threaded on pillar.

M, mirror, in which lower surface of fluorescent plate can be seen when front of camera is opened as in the diagram.

MU, measuring unit of Photovolt photometer.

PIL, pillars which limit the motion of the lower plate of the scanner.

PC, plate compartment.

PP, battery power pack for photomultiplier tube.

S, scanner.

SH, shaft on which upper plate rotates.

SR, shutter to cut off ultraviolet light. On its upper surface is a mirror which reflects light from the tungsten lamp into the microscope, when the shutter is closed.

ST, adjustable stop to limit motion of the upper plate of the scanner.

SU, search unit of Photovolt photometer.

SU AP, search unit aperture in the upper plate, through which the microscopic image is projected to the photosensitive surface of the phototube.

T, telescope of focuser.

UP, upper plate of scanner.

UPL, upper plate lock.

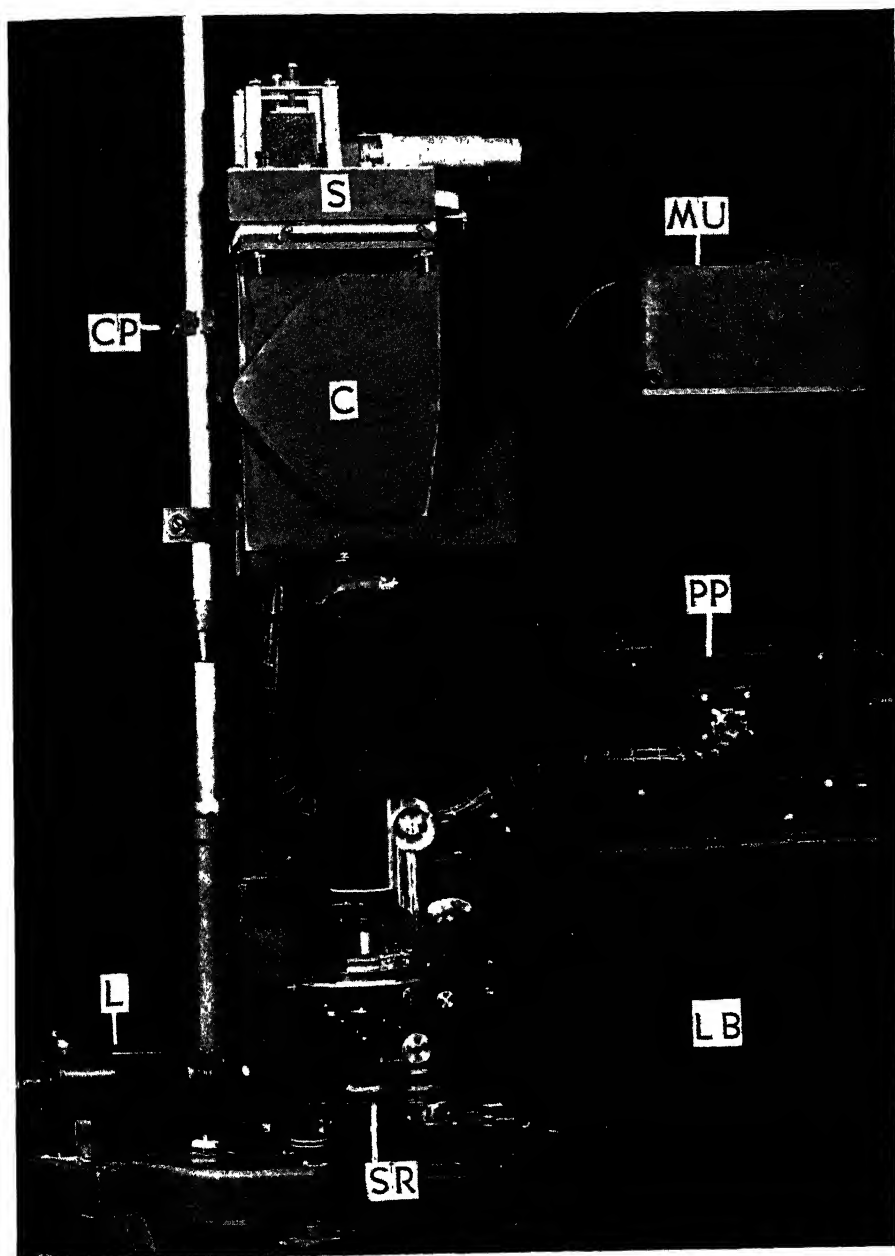


FIG. 1

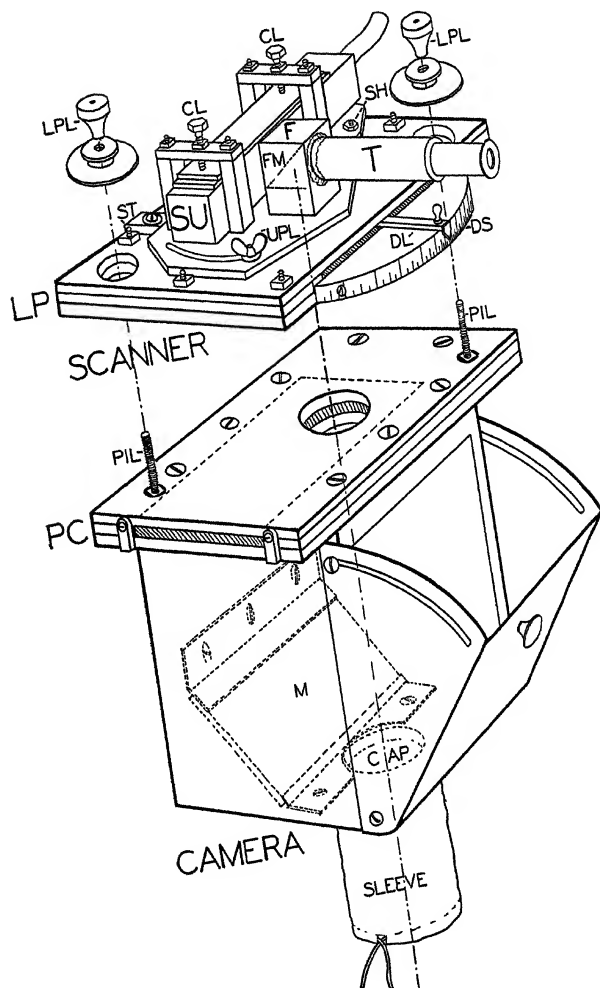


FIG. 2

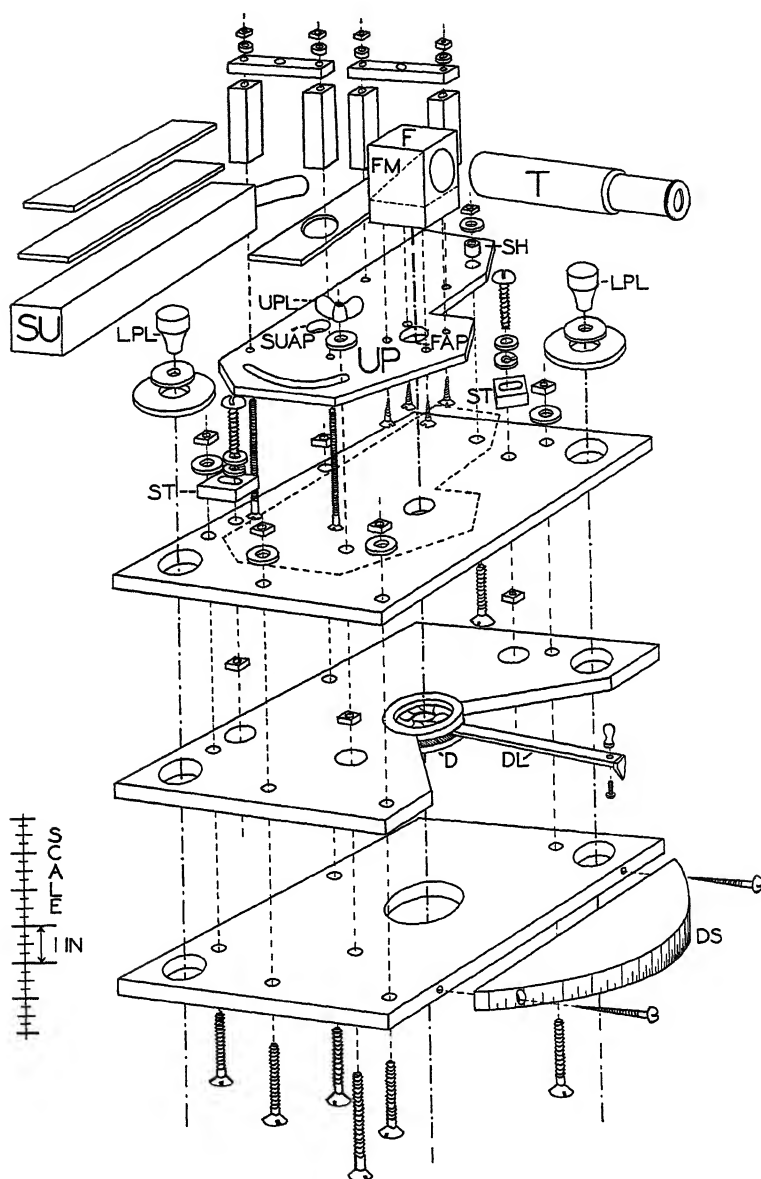


FIG. 3

THE STABILITY OF BACTERIAL VIRUSES IN SOLUTIONS OF SALTS*

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Burnet and McKie (1) in 1930 published a report on balanced salt action as manifested in bacteriophage phenomena. They found that *coli* and staphylococcal bacteriophages when diluted in 0.1 N solutions of sodium, potassium, and ammonium salts were much more susceptible to the inactivating effects of temperature (60°C.) than were the same phages diluted in broth. The addition of a small amount of calcium, magnesium, or barium salt partially or completely prevented this inactivation. They interpreted this phenomenon as another example of the physiological ion antagonism studied by Ringer, Jacques Loeb, and many other physiologists. Gratia (2) has drawn similar conclusions from experiments on the stability of a *megatherium* phage in salt solutions.

In a study of the properties of the *coli*-dysentery phages in chemically defined media we have found that these phages exhibit a similar phenomenon, which, however, cannot be explained on the basis of ion antagonism. The present paper is a report on the kinetics of inactivation of phages in the presence of various ions and on the effect of the environment on the rate of inactivation.

Materials and Methods

The group of seven *coli*-dysentery phages studied by Demerec and Fano (3) was used. Certain properties of this group of bacterial viruses have been summarized by Delbrück (4). These phages were grown on *Escherichia coli*, strain B, in a chemically defined medium containing per liter 1 gm. NH_4Cl , 0.1 gm. MgSO_4 , 1.5 gm. KH_2PO_4 , 3.5 gm. Na_2HPO_4 , and 9 gm. lactic acid. The medium was adjusted to pH 6.5 by the addition of NaOH . Since phage T5 is not produced in the absence of calcium ion, calcium chloride to a final concentration of 0.001 M was added when preparing stocks of this phage. All phage stocks used contained between 10^8 and 10^{10} plaque-forming particles per ml. All phage assays were made on strain B of *E. coli* using the agar layer technique of Gratia as modified by Hershey (5). The plating medium was Difco nutrient agar to which 0.5 per cent of sodium chloride was added. The broth used in certain experiments was Difco nutrient broth with 0.5 per cent of sodium chloride. The pH of these broth-containing media was 6.8.

All glassware used in this study was cleaned with acid dichromate and repeatedly rinsed with hot distilled water since, as will be shown later, very small amounts of

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

salts markedly affect the results. Solutions used in the kinetic studies were made up in distilled water redistilled from an all glass apparatus.

EXPERIMENTAL

Preliminary Experiments.—In connection with certain experiments with phage T5 in a chemically defined medium, sodium citrate to a concentration of 0.001 M was added to form a weakly ionized complex with small amounts of calcium ion present in the medium. It was found that under these conditions the infectivity of the phage was rapidly lost. The stability of phage T5 was then tested in various media. The phage was diluted in 0.01 M phosphate buffer at pH 7.00 to a concentration of 5×10^4 infectious particles per ml. It was then further diluted 1/10 in 0.01 M phosphate buffer, in buffer plus 0.002 M

TABLE I

The Stability of Phage T5 at 37° C. and pH 7.0 When diluted in 0.01 M Phosphate Buffer, and in Buffer Plus Citrate or Calcium

Diluent	Phage assay at time		
	0	30 min.	2 hrs.
0.01 M PO_4 buffer.....	380	353	158
Same + 0.001 M CaCl_2	333	438	376
" + 0.002 M citrate.....	44	2	0

The phage assays are the number of plaques formed when 0.1 ml. samples are plated on nutrient agar.

sodium citrate, and in buffer plus 0.001 M calcium chloride. These solutions were incubated at 37°C. and assayed at intervals for phage activity with the results shown in Table I.

From the results in Table I it is evident that the phage is stable in the presence of calcium ion, loses activity slowly in 0.01 M phosphate buffer, and is very rapidly inactivated in the presence of citrate ion. In the latter case almost 90 per cent of the phage is inactivated before the zero time sample could be taken, that is in less than a minute.

Prevention of Inactivation by Divalent Cations.—From other experiments it became evident that the inactivation of phage T5 was not attributable to the anions present, that the effect of citrate was due to removal of calcium and magnesium ions through complex formation, and that the inactivation of T5 could be prevented by any one of a number of divalent cations. To demonstrate the latter point a diluent was used which contained 0.001 M phosphate buffer at pH 7.0, 0.15 M sodium chloride, and 0.1 mg. of Eastman salt-free gelatin per ml. to prevent surface inactivation of the virus in long term experiments (6). To this diluent various salts were added and the pH was checked. The

phage was diluted to a concentration of 10^6 infectious particles per ml. in the diluent, then further diluted 1/100 in the diluent plus various additions. The phage solutions were incubated at 37°C . and sampled at intervals for assay with the results shown in Table II.

From the data in Table II it is evident that all the divalent metals tested at a concentration of 10^{-3} M have a definite protective effect against the inactivation with the exception of lead and mercuric salts. Calcium ion at a concentration of 10^{-4} M still exerts a considerable protective effect, but protection is not evident under these conditions with calcium at 10^{-5} M concentration.

TABLE II

The Effect of Various Divalent Cations on the Inactivation of Phage T5 in 0.15 N Sodium Ion at pH 7.0 and 37°C .

Diluent	Phage assay at time		
	0	2 hrs.	24 hrs.
Diluent alone.	736	2	0
" + 10^{-3} M Ca^{++}	778	436	384
" + 10^{-4} M Ca^{++}	932	300	25
" + 10^{-5} M Ca^{++}	960	2	0
" + 10^{-3} M Ba^{++}	760	441	359
" + 10^{-3} M Sr^{++}	697	321	288
" + 10^{-3} M Mg^{++}	632	393	369
" + 10^{-3} M Mn^{++}	468	335	364
" + 10^{-3} M Co^{++}	706	310	297
" + 10^{-3} M Ni^{++}	699	321	298
" + 10^{-3} M Zn^{++}	594	279	265
" + 10^{-3} M Cd^{++}	376	268	171
" + 10^{-3} M Cu^{++}	622	282	55
" + 10^{-3} M Pb^{++}	550	1	0
" + 10^{-3} M Hg^{++}	430	5	0

Kinetics of Inactivation in the Presence of Low Concentrations of Sodium Ion.—

Since the inactivation of phage T5 in solutions of sodium ion seemed to be rather sensitive to the presence of small amounts of other ions, it was deemed desirable to purify specially some salt of sodium. A concentrated aqueous solution of reagent grade trisodium phosphate was boiled for some time, yielding a considerable amount of amorphous precipitate which was removed by filtration. The solution was allowed to crystallize, and the crystals subjected a second time to the same treatment. The twice recrystallized salt remained clear and colorless on boiling in concentrated solution. A weighed sample of the twice recrystallized salt was dissolved in redistilled water, adjusted to a pH of 7.0 by the addition of redistilled reagent grade HCl, and diluted with water to a sodium concentration of 1 N. This solution was further diluted with

water to give a series of solutions of known sodium ion concentration which were used as diluents for phage T5. The phage was diluted in these diluents to a concentration of 10^4 infectious particles per ml., incubated at 37°C ., and sampled at intervals for assay with the results shown in Table III.

From the data of Table III it would seem that phage T5 is relatively stable at concentrations of sodium ion of 0.4 N or higher. However, at sodium ion concentrations of 0.2 N and 0.1 N the phage is rapidly inactivated. The kinetics of inactivation are of the first order with a velocity constant of 0.08 min.^{-1} for 0.2 N sodium ion and 0.9 min.^{-1} for 0.1 N sodium ion at 37°C . and pH 7.0.

TABLE III

The Inactivation of Phage T5 in Various Concentrations of Sodium Phosphate at 37°C . and pH 7.0

Time <i>min.</i>	The No. of plaque-forming particles surviving at the indicated times in diluent containing				
	0.8 N Na ⁺	0.6 N Na ⁺	0.4 N Na ⁺	0.2 N Na ⁺	0.1 N Na ⁺
1	1541	1465	1781	1304	461
3	—	—	1413	971	81
5	—	—	—	856	10
10	—	—	—	730	1
20	—	—	—	226	3
30	—	—	—	191	2
60	1340	1586	1354	30	2

The dashes indicate that the plates concerned were not counted but appeared to have about the same number of plaques as those in the same series that were counted. The rate of inactivation is significant only at sodium ion concentrations of 0.2 N and 0.1 N.

To insure that the inactivation of T5 in the presence of low concentrations of the neutralized Na_3PO_4 was not merely a peculiarity of the particular lot of salt chosen these experiments were repeated with NaCl. Reagent grade sodium chloride was twice recrystallized from a saturated solution in distilled water by the addition of alcohol. A normal solution of this salt was prepared in redistilled water and appropriately diluted in redistilled water and 0.01 N phosphate buffer, prepared as described above, so that the final solution contained a known concentration of sodium chloride and sodium phosphate buffer at 0.001 N and pH of 6.0. The T5 phage was diluted in these solutions at room temperature to a concentration of 3×10^6 infectious particles per ml. and then further diluted 1/100 in the same solution at 37°C . and sampled at intervals for assay. At concentrations of sodium chloride of 0.8 N and 0.4 N the inactivation of T5 was inappreciable in 1 hour. The first order velocity constants for inactivation of T5 were 0.11 min.^{-1} at 0.2 N NaCl and 1.0 min.^{-1} at 0.1 N NaCl. Experiments were carried out also at sodium ion concentrations of 0.075 N, 0.05 N, 0.026 N,

0.011 N, 0.006 N, and 0.001 N, the first order velocity constants over this entire range varying randomly between 1 min.^{-1} and 2 min.^{-1} .

The Effect of Temperature on the Inactivation of Phage T5 in Dilute Sodium Chloride.—The diluent used in these experiments contained 0.1 N sodium chloride and 0.001 N sodium phosphate buffer at pH 6.0, both salts being twice recrystallized as described above and dissolved in redistilled water. Phage T5 was diluted to about 2×10^6 particles per ml. in the diluent at 0°C . This was

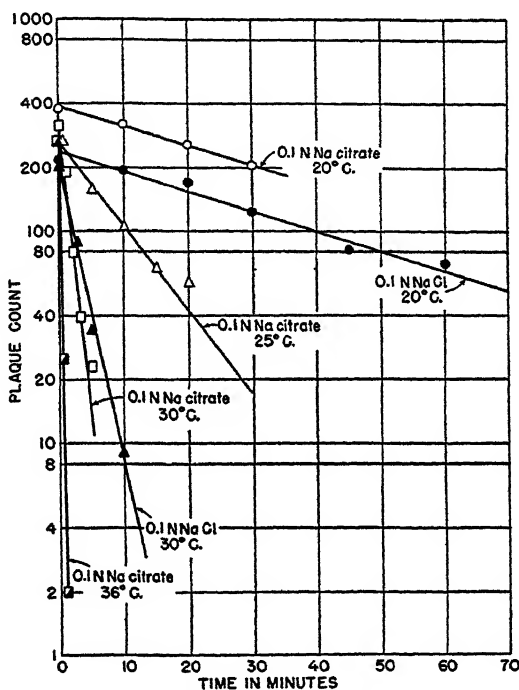


FIG. 1. Plaque counts per sample plotted on a logarithmic scale against the time of sampling for phage T5 in 0.1 N NaCl or 0.1 N Na citrate at various temperatures.

then diluted 1/100 in the diluent which had been already equilibrated at the temperature chosen for the experiment. Zero time for the experiment was taken as the moment at which this 1/100 dilution was made. At appropriate intervals samples were removed and diluted 1/10 in broth to stop the inactivation, since phage T5 is not inactivated at an appreciable rate in broth below 50°C . At a convenient time 0.1 ml. samples of these broth dilutions were plated by the agar layer method for assay. The zero time counts were taken from a direct assay of the phage in the diluent at 0°C .

The data of these experiments are given graphically in Fig. 1, in which the plaque counts of the samples are plotted on a logarithmic scale against the

time of sampling. Each point represents the count of a single plate. Also included in Fig. 1 are data for the inactivation of phage T5 in 0.1 *N* sodium citrate adjusted to pH 6.0 with HCl. The rates of inactivation are probably not significantly different whether the anion involved is citrate, phosphate, or chloride.

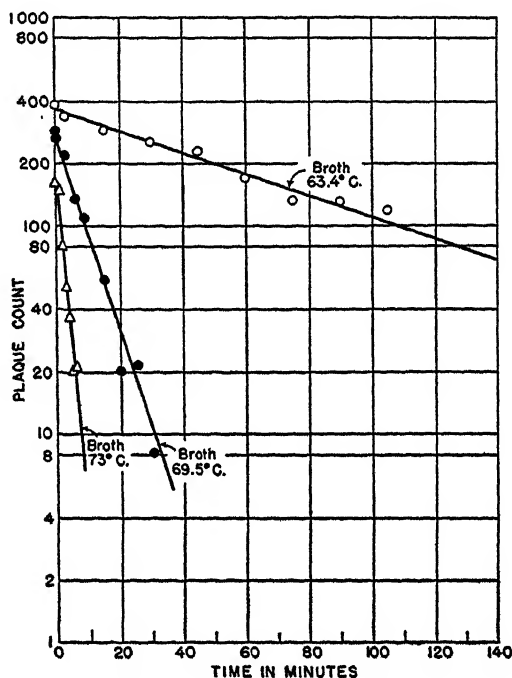


FIG. 2. Plaque counts for the inactivation of phage T5 in broth at various temperatures as a function of time.

In all cases the plaque count decreases as an exponential function of time with no initial lag. The inactivation of phage T5 under these conditions follows the kinetics of a first order reaction, as is usual in the inactivation of viruses whether by heat, by irradiation, or by chemical action. The first order velocity constants were calculated from the slope of the inactivation curve for each experiment and are presented in the form of an Arrhenius plot in Fig. 6.

As noted above, phage T5 is markedly more stable in broth than in 0.1 *N* sodium salts. However, at temperatures above 60°C. this virus in broth is inactivated at readily measurable velocities. The plaque counts as a function of time of exposure to three different temperatures are given in Fig. 2. The rate of inactivation is an exponential function of time, and the first order velocity constants calculated from the slopes of the curves are presented in the form of an Arrhenius plot in Fig. 6.

The Effect of Magnesium Ion on the Rate of Inactivation of Phage T5 in Dilute Solutions of Sodium Salts.—Since the marked protective effect of various divalent cations against the inactivation of phage T5 in 0.1 N sodium salts had been noted (Table II), it was decided to determine the effect of various concentrations of magnesium ion in the presence of 0.1 N NaCl and 0.001 N phosphate buffer on the velocity of inactivation of phage T5 at various temperatures. The results of these experiments are given in graphical form in Figs. 3, 4, and 5. In all cases the kinetics of inactivation are of the first order. The addition of small

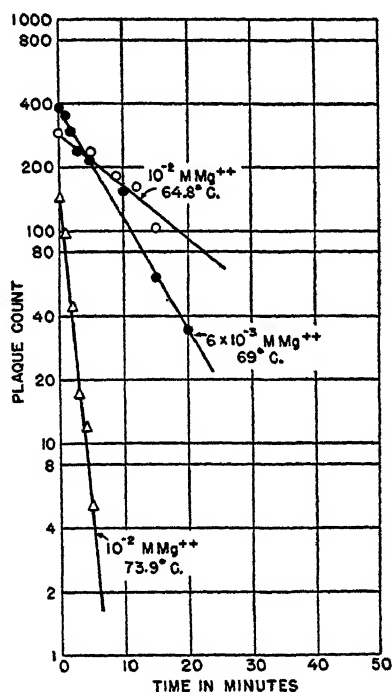


FIG. 3. Plaque counts for the inactivation of phage T5 in 0.1 N NaCl plus added magnesium at various temperatures as a function of time.

amounts of magnesium ion to the saline diluent increases the stability of phage T5 so that the temperature must be raised to bring about inactivation at a measurable rate. The first order velocity constants for each experiment have been calculated and are given in Fig. 6 in the form of Arrhenius plots.

In Fig. 6 the logarithms of the first order velocity constants for inactivation of phage T5 are plotted against the reciprocal of the absolute temperature at which the inactivation was carried out (Arrhenius' method of demonstrating the relationship between reaction rate and temperature). The lines have been drawn through the points, with the result that the slopes of the curves vary in a

random manner. This is misleading because there is reason to believe that all the curves should be parallel to the broth curve with the exception of the curves which contain no added magnesium. The rate of inactivation of phages in salt diluents is markedly affected by traces of impurities such as detergents and oxidizing agents and by ions such as magnesium or citrate. For this reason any single rate determination in salt diluents is subject to some uncertainty. This

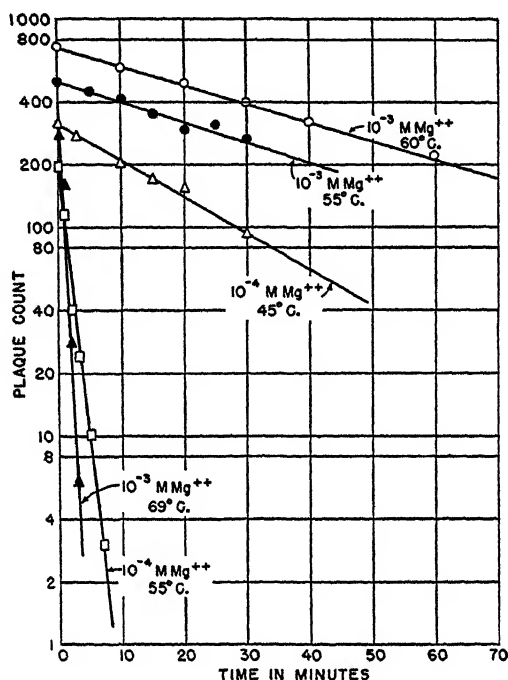


FIG. 4. Plaque counts for the inactivation of phage T5 in 0.1 N NaCl plus added magnesium at various temperatures as a function of time. Note that the time scale for the experiment with 10^{-3} M magnesium ion at $55^{\circ}C$. is to be multiplied by six, that is the ten minute sample was taken at 1 hour, etc.

is not true of the experiments in broth since the properties of this diluent are not affected by traces of impurities. For these reasons the Arrhenius plots in Fig. 6 unduly exaggerate the experimental errors inherent in work of this kind. Nonetheless the relative positions of the curves along the temperature axis adequately demonstrate the marked effect of small amounts of magnesium salts on the stability of phage T5.

In order to compare the effects of varying magnesium ion concentrations on the velocity constants for inactivation of phage T5, the Arrhenius plots of Fig. 6 have been extrapolated to a common temperature ordinate. A temperature of

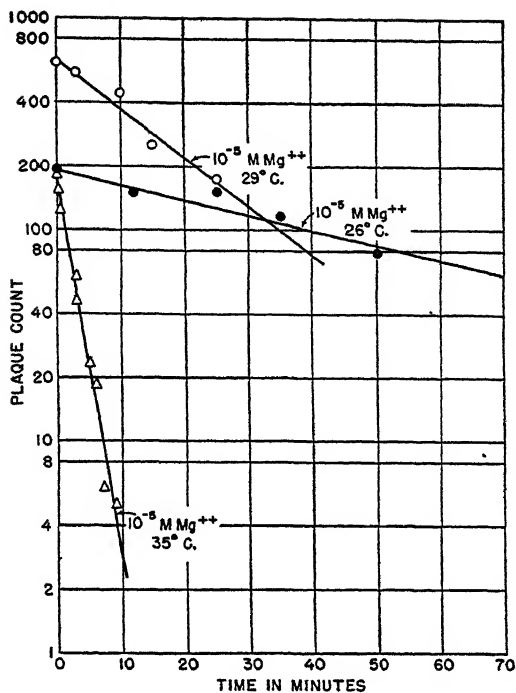


FIG. 5. Plaque counts for the inactivation of phage T5 in 0.1 N NaCl plus added magnesium at various temperatures as a function of time.

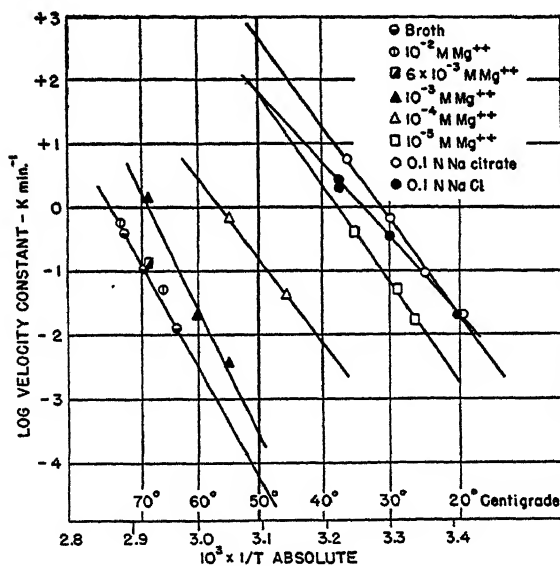


FIG. 6. The log of the first order velocity constants for inactivation of phage T5 as a function of temperature and of the magnesium concentration.

50°C. was chosen since it is near the midpoint of the experimentally available temperature range and hence would require less extrapolation of the Arrhenius plots. From the values of the intercepts it is evident that the rate of inactivation of phage T5 in 0.1 N saline at 50°C. is at least one million times that in broth at the same temperature, and that any velocity between these two limits can be achieved by adding a suitable concentration of magnesium salt. The relationship between velocity constant and magnesium concentration is expressed graphically in Fig. 8 in which the log of the first order velocity constant at 50°C. is plotted against the log of the magnesium concentration. The slope of this line is -2.7 which means that the velocity of inactivation is inversely proportional to the second or third power of the magnesium concentration. Since the protective effect of magnesium is neutralized by an excess of citrate ion, it would appear that the effective agent is magnesium ion rather than the total amount of magnesium salt present. Therefore, a more precise quantitative determination of the effect of magnesium ion on phage stability would require an independent assay of the magnesium ion activity in these highly diluted solutions.

The Effect of Increased Concentrations of Sodium Ion on the Inactivation of Phage T5.—As was noted in Table III, higher concentrations of sodium ion seemed to protect phage T5 from temperature inactivation in the same way as did the addition of divalent cations. The results of the experiments involving various concentrations of sodium ion are included in Fig. 7 in which the log of the first order velocity constant for inactivation of phage T5 is plotted against the reciprocal of the absolute temperature at which the experiment was done. It is evident from these Arrhenius plots that an increase in the concentration of sodium ion results in an increase in the stability of the phage to temperature, the phage being as stable in 2 N NaCl as it is in broth. In Fig. 8 is included a plot of the first order velocity constants extrapolated to 50°C. as a function of the concentration of sodium ion in the medium. From this plot it would appear that the rate of inactivation of phage T5 at 50°C. is inversely proportional to the fifth or sixth power of the sodium ion concentration. The precise relationship between the velocity constants and the sodium ion concentration is uncertain because of the experimental errors involved. The relationship may not be a simple one because changing the sodium concentration from 0.1 N to 2 N involves a large change in ionic strength, the effect of which on the inactivation of phage is not known. It is, however, certain that the effect of sodium ion on the velocity constant is quantitatively different from the effect of magnesium ion which renders it highly unlikely that the protective effect of higher concentrations of sodium ion is due to contamination with small amounts of magnesium ion.

The Relative Stability of Other Phages in Broth and Dilute Saline.—Data for the inactivation of phages T1, T4, and T7 in broth and in 0.1 N sodium chloride

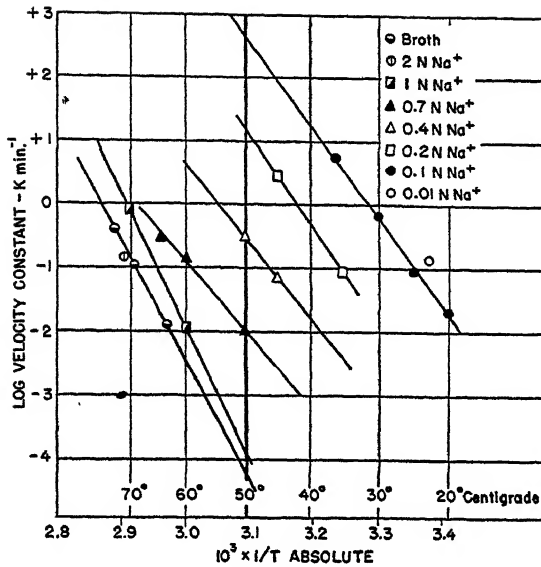


FIG. 7. The log of the first order velocity constants for inactivation of phage T5 as a function of temperature and of the sodium concentration.

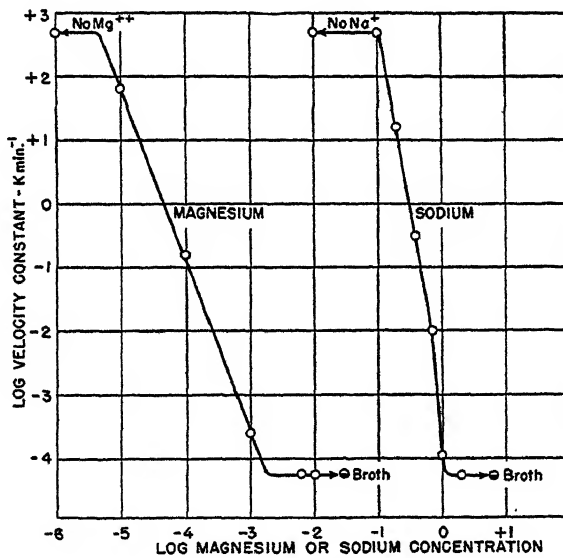


FIG. 8. The log of the first order velocity constants for inactivation of phage T5 at 50°C. as a function of the log of the magnesium or sodium concentrations in moles per liter.

are presented in Fig. 9. In all cases it may be seen that the phage is more stable in broth than in saline although with phage T4 the difference is much

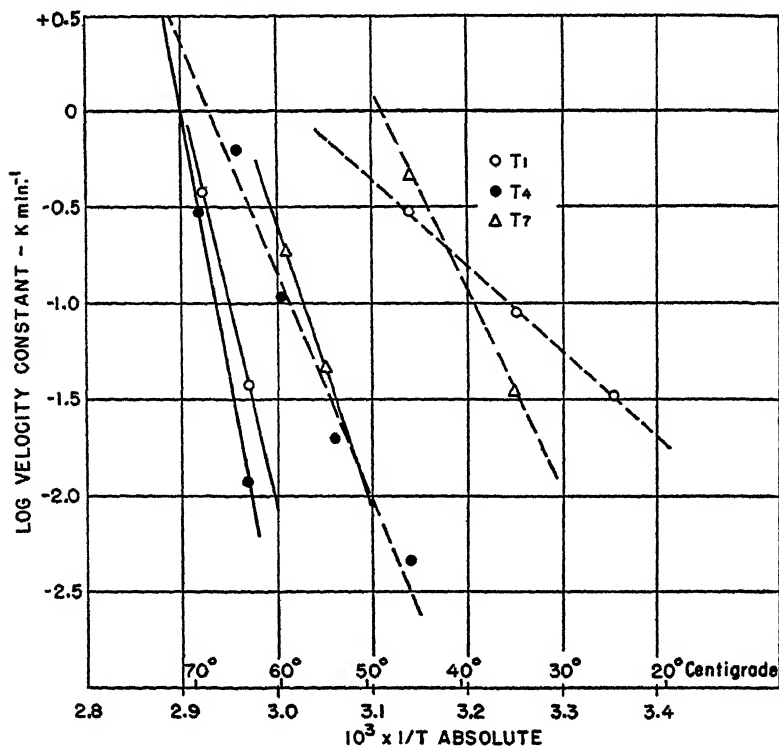


FIG. 9. The log of the first order velocity constants for the inactivation of phages T1, T4, and T7 as a function of temperature and of the medium. The solid lines represent inactivation in broth, while the dashed lines represent inactivation in 0.1 N sodium chloride.

less marked than in the other cases. Phage T3 resembles T7 and phage T6 is similar to phage T4 when subjected to heat in the two media.¹

¹ It is probable that phage T2 is similar to T4 and T6 but it was not possible to study this phage because the inactivation did not follow the kinetics of a first order reaction. The inactivation proceeded very slowly at first, then accelerated, eventually becoming approximately first order when 90 per cent of the infectivity was gone. The velocity of inactivation of the residual 10 per cent of the phage was very close to that of T4 and T6 at the same temperature. This type of behavior might be expected if the virus consisted largely of pairs or small clumps of virus particles in which the inactivation of one member of the pair or clump would not result in a decrease in the number of the infective centers. This supposition is born out by the fact that phage

The Arrhenius constants for the inactivation of the phages may be calculated by multiplying the slopes of the curves in Figs. 6, 7, and 9 by 4.58. These constants for experiments in broth are given in Table IV.

It may be seen from Table IV that the Arrhenius constants for heat inactivation of phages in broth are of the order of magnitude of those determined for the heat denaturation of proteins and for the heat inactivation of enzymes and of bacterial toxins.

TABLE IV

The Arrhenius Constants for the Heat Inactivation of Phages T1, T4, T5, and T7 in Broth

Phage	Arrhenius constant in broth
T1	106,000
T4	131,000
T5	86,000
T7	77,000

The effect of hydrogen ion concentration on the rate of inactivation of phage T5 in 0.1 N NaCl at 37°C. was also studied. Over the range from pH 5.5 to 7.5 at least, the rate of inactivation seemed to be independent of hydrogen ion concentration, but below 5 or above 9, the rate of inactivation was greatly accelerated. The effect of pH on the rate of inactivation of the other phages has not been measured.

DISCUSSION

The *coli*-dysentery phages of the T group with the possible exception of T2 are inactivated by heat at an exponential rate. With T5 the kinetics are of the first order whether the inactivation is at 20°C. in 0.1 N sodium salts, or at 70°C. in broth. The inactivation of bacteriophages by other agencies such as x-radiation (7), ultraviolet irradiation (8), heat (12), and surface denaturation (6) has also been found to follow the kinetics of a first order reaction. The destruction of infectivity of influenza virus by heat was found to be a first order reaction (9). It would seem from these examples that in general the destruction of the infectivity of a virus follows the kinetics of a first order reaction regardless of the agent responsible for the destruction. Exceptions to this generalization as in the case of phage T2 may probably be accounted for by

T2 diluted in broth at 37°C. actually shows an increase in the number of infective centers often doubling in titer in the course of an hour. An investigation of the conditions required for the dissociation of phage T2 in various salt solutions would be necessary before the kinetics of inactivation of T2 could be studied.

the clumping of two or more virus particles to form a single infective unit with increased resistance to inactivation (10).

From the data presented in this paper it is evident that the bacteriophages studied are markedly less stable in the presence of 0.1 *N* sodium ion than they are in broth. The lowered stability in dilute sodium ion is manifested by a lower temperature at which a given first order velocity constant for inactivation is reached. Since the difference in stability seemed to be most marked in the case of the phage T5, most of the work has been done with this phage. The addition of a variety of divalent cations at a concentration of 10^{-3} *M* to a salt medium containing 0.15 *N* sodium ion resulted in a marked increase in the stability of phage T5 at 37°C. In a more quantitative study in which various concentrations of magnesium ion were added to a salt solution containing 0.1 *N* sodium ion it was found that the stability of phage T5 increased with increasing concentrations of magnesium ion reaching an optimal stability indistinguishable from that in broth at a magnesium concentration of 6×10^{-3} *M*. Since the Arrhenius plots for the inactivation of phage T5 in 0.1 *N* sodium ion and in the presence of 10^{-2} *M* magnesium ion converge, it would appear that the stabilizing effect of magnesium ion decreases with increasing temperature becoming zero at the point of intersection. In the case of phage T5 the velocity of inactivation at the point of intersection is too high to be measured. With T4, however, the point of intersection of the two curves is about 74°C. and at a velocity constant of about 5 min.⁻¹ which corresponds to a rate of inactivation just a little faster than can be measured by our techniques. It is conceivable that with other viruses the point of intersection would occur at velocities of inactivation too low to be conveniently measured, in which case there would be no detectable difference in stability of the virus whether tested in broth or in salt solutions.

When the log of the velocity constant for inactivation of phage T5 at 50°C. is plotted against the log of the magnesium ion concentration an S-shaped curve is obtained, the linear portion of which has a slope between -2 and -3 (Fig. 8). This means that over the range of the linear portion of the curve, the first order velocity constant for inactivation is inversely proportional to the second or third power of the magnesium ion concentration.

This relationship might be most simply explained by assuming that the phage reacts with Mg^{++} ion to form a weakly dissociated complex in accordance with the equation: $\text{phage} + n \text{Mg}^{++} \rightarrow \text{phage-Mg}_n$. The free phage undergoes irreversible inactivation at 50°C. at a rate which is about 10^6 -fold greater than the rate of inactivation of the phage-magnesium complex. The actual rate of inactivation of phage in any given concentration of magnesium ion is largely determined by the concentration of free phage which in turn is determined by the magnesium ion concentration in accordance with the equation. The phage-magnesium complex must dissociate to yield free phage and mag-

nesium ion at a rate which is at least as great as the rate of inactivation of phage under the experimental conditions.

It would appear from the data summarized in Fig. 8 that the phage will also form a complex with sodium ion, and that the dissociation constant for such a complex must be much higher than that for the magnesium complex. Since the velocity constant of inactivation is inversely proportional to the fifth or sixth power of the sodium ion concentration, it would seem that the phage is able to associate more sodium ions than magnesium ions. The situation in the case of the sodium ion, however, is so complex, involving marked changes in ionic strength as well as temperature when the sodium ion concentration is increased, that mathematical analysis of the available data would have little value.

Phage T5 is one of the phages (11) which requires relatively high concentrations of calcium ion in the medium for reproduction to take place. The remaining phages of the T group require much less or no calcium ion for growth, and the host cell, *E. coli*, also appears to require little or no calcium ion for multiplication. For instance plaque formation with phage T5 on nutrient agar plates is completely inhibited by 1 per cent sodium citrate whereas growth of the host cell is not appreciably affected. The calcium ion is not needed for adsorption of phage T5 to the host cell, but is required for some later step in the life cycle of the virus. The calcium requirement cannot be met by magnesium ion or other common cations. Since the calcium requirement is specific for the virus rather than for the host cell, it is likely that the calcium ion takes part in some metabolic reaction peculiar to phage multiplication, perhaps activating some enzyme which is part of the virus. The ability of the virus T5 to form complexes with various cations leading to increased heat stability may possibly be related to the requirement of calcium for some metabolic purpose.

SUMMARY

1. The seven bacterial viruses of the T group, active against *E. coli*, are much more rapidly inactivated by heat when suspended in 0.1 N solutions of sodium salts than when suspended in broth.

2. The kinetics of this inactivation whether in salt solutions or in broth are those of a first order reaction.

3. The rate of inactivation of phage T5 in 0.1 N NaCl at 37°C. can be greatly decreased by the addition of 10^{-3} M concentrations of such divalent cations as Ca, Mg, Ba, Sr, Mn, Co, Ni, Zn, Cd, and Cu.

4. An increase in the cation concentration in the suspending medium results in an increase in the stability of phage T5 to the inactivating effects of temperature.

5. The hypothesis is proposed that the increase in stability of phage T5 in

the presence of various cations is the result of complex formation between the phage and the metal ion.

The author wishes to acknowledge the assistance of Miss Nancy J. Collins in the experimental work.

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STUDIES ON THE MECHANISM OF ACTION OF IONIZING RADIATIONS

II. INHIBITION OF SULFHYDRYL ENZYMES BY ALPHA, BETA, AND GAMMA RAYS

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In the preceding paper (1) it was shown that dilute aqueous solutions of sulfhydryl enzymes are inhibited by small doses of x-rays by oxidation of the —SH groups of the protein moiety. Hardly any studies have been made on the effect of other ionizing radiations. Northrop (2), who studied the inactivation of crystalline pepsin by beta and gamma rays from radium, reported that inactivation required large amounts of radiation. Presented in this paper are experiments on the effect of alpha, beta, and gamma radiations on the activity of two crystalline sulfhydryl enzymes, phosphoglyceraldehyde dehydrogenase and urease. Enzyme inhibition by these radiations was produced by the same mechanism as that of x-rays; *i.e.*, oxidation of the —SH groups by the products of water irradiation.

EXPERIMENTAL

The water was purified with the same precautions as those indicated in the preceding paper (1).

The source of alpha radiation was a solution of citrate buffer containing 30 microcuries per cc. of polonium. One cc. was diluted to 10 cc. with 0.2 M phosphate buffer, pH 7.0. The irradiated tubes received 0.4 cc. of this solution to a total of 2.1 cc. The final polonium concentration was 0.57 microcuries per cc. This amount of radiation was calculated to give 180 r per day per cc.

The source of beta radiation was a solution of $\text{Sr}^{90}\text{Cl}_2$ containing 250 microcuries per cc. One cc. was diluted to 11.25 cc. with citrate buffer, pH 7.0, and this in turn was diluted ten times more with 0.2 M phosphate buffer, pH 7.0. For irradiation, 0.4 cc. of this solution was added to a total volume of 2.1 cc. The final concentration of Sr^{90} was 0.42 microcuries per cc. This amount of radiation was calculated to give 14.6 r per day per cc.

The source of gamma rays was a 1 gm. sample of radium enclosed in a brass tube with a long piece of silk fishing line attached to one end. This was kept in a lead brick cave. The line led through a hollow aluminum tube to a pulley fastened to the ceiling (Fig. 1). Before the experiments started, the source was raised to the ceiling and the aluminum tube shifted from the cave to a lusteroid test tube placed in the center of the holder containing the test tubes to be irradiated. The source

was then lowered into position, and the time of irradiation was measured with a stop watch. The sample holder was made up of two round half-inch pieces of lucite with a hole in the middle of each to hold the tube in which the source was stationed. A series of holes at 5 cm. radius from the center held the pyrex tubes containing the enzyme. One hole of this set was enlarged to accommodate a test tube that held the Victoreen dosimeter (Fig. 2). A series of tests with a 250 r capacity dosimeter indicated a rate of gamma ray emission of 4.5 r per minute at 5 cm. distance. The holder fitted smoothly into a half-gallon Dewar flask that contained sufficient cracked

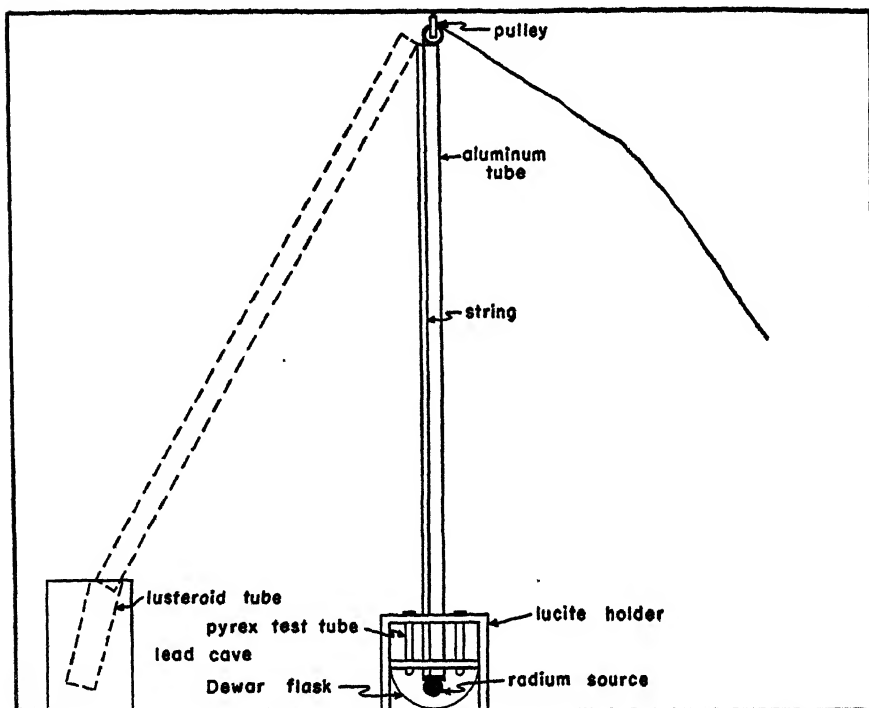


FIG. 1. Apparatus for the irradiation of enzymes with gamma rays.

ice and water to surround the solutions in the test tubes. A piece of wood in the bottom of the central tube maintained the level of the source at the same height at the bottom of the tubes containing the enzyme.

Urease was prepared from arlico jack bean meal by a modification of the method of Hellerman *et al.* (3). The first crop of crystals was dissolved in 1×10^{-2} M neutralized glutathione instead of water. Urease activity was determined by a modification of the method of Van Slyke and Archibald (4). It was found that addition of 0.1 cc. of 1 M glycine to the urea-phosphate solution acted as an effective substitute for the egg albumin recommended by these authors. Quantitative recoveries of added urease were easily secured when glycine was added to the substrate. The

determination of urease activity was carried out at the temperature of the laboratory (24–28°); the correction factor of Van Slyke and Archibald was utilized in the calculation of urease units. The incubation period was 15 minutes, followed by addition of saturated carbonate and aeration for 30 minutes into 4 per cent boric acid. The liberated ammonia was titrated with 0.02 *N* HCl, using the mixed indicator of Sobel *et al.* (5).

The Sulfhydryl Groups in Phosphoglyceraldehyde Dehydrogenase.—The presence of —SH groups on this enzyme was concluded from Rapkine's experiments in muscle suspensions (6), but it was necessary to demonstrate their presence

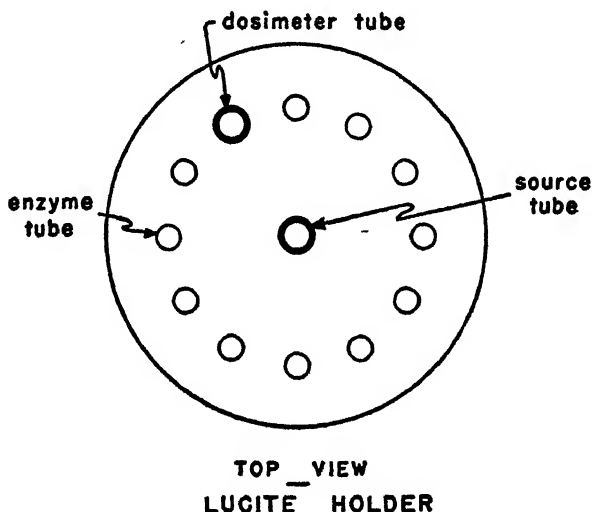


FIG. 2. Lucite holder where the test tubes containing enzyme solutions are kept when irradiated with gamma rays.

in the crystalline enzyme. A solution of the enzyme (17 micrograms) in phosphate buffer, pH 7 ($\mu = 0.2$) was treated with increasing amounts of *p*-chloromercuribenzoate (0.001 *M*). At the end of 30 minutes, the enzyme activity of an aliquot was measured. Enzyme inactivation was proportional to *p*-chloromercuribenzoate addition (Table I). From extrapolation of these activity titrations, it was calculated that 1 gm. of enzyme contained 0.79 mM —SH groups. Reactivation of the enzyme was attempted by addition of glutathione immediately before the determination of enzyme activity. Under these conditions glutathione failed to produce complete reactivation of the enzyme. This is the first case where inhibition by a mercaptide-forming agent was not reversed on addition of glutathione. The —SH groups of native and duponol PC-denatured enzyme were also determined with Anson's ferricyanide method (7) (Table II). From these titrations it can be concluded that

the native protein contains 0.23 mM and the denatured protein, 0.82 mM of —SH groups per gm. From a comparison of the titration of enzyme activity with *p*-chloromercuribenzoate with the titration of the total —SH groups in the denatured protein, it may be concluded that most of the —SH groups of the protein (93 per cent) are necessary for enzyme activity.

Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase.—Polonium was chosen as the source for alpha ray emission because it emits alpha rays of energy 5.298 e.m.v.¹ with practically no other radiation. The test tubes

TABLE I
*Inhibition of Phosphoglyceraldehyde Dehydrogenase by p-chloromercuribenzoate
Effect of Glutathione*

<i>p</i> -Cl-Hg benzoate <i>cc. of 1 × 10⁻³ M</i>	<i>K</i> values		Inhibition <i>per cent</i>
	Without GSH <i>× 10⁵</i>	With GSH <i>× 10⁵</i>	
0	7.5	7.5	
0.05	4.8	4.8	36
0.10	1.6		79
0.12	0.7		90
0.20	0	0	Complete

TABLE II
The Sulfhydryl Groups of Native and Denatured Phosphoglyceraldehyde Dehydrogenase
Sulfhydryl groups measured by ferricyanide titration. The figures give micromoles —SH per gm. protein.

Sample	SH groups in protein	
	Native <i>micromoles</i>	Denatured <i>micromoles</i>
I	0.246	0.817
II	0.22	0.80
III	0.22	0.81

containing enzyme and polonium were kept with the control test tubes at the temperature of cracked ice in a room at 3°. The enzyme activity of the control solutions remained unimpaired for 6 days, while the activity of the solutions containing polonium decreased steadily, so that at the end of 6 days enzyme activity had entirely disappeared (Table III). These experiments demonstrate that alpha rays are as effective as x-rays in inhibiting this sulfhydryl enzyme. Evidence that this inhibition is partly due to oxidation of the —SH groups of the protein was obtained by the measurement of enzyme activity after the addition of glutathione (0.01 M neutralized glutathione added simultaneously

¹ e.m.v. signifies 10⁶ e.v. (electron volts).

to the control and to the irradiated samples); a partial reactivation—from 26 to 32 per cent—was always obtained (Table IV).

When alpha rays irradiate water, a definite amount of H_2O_2 is produced, which according to Frilley (8) is 0.54 molecules per ion pair in the solution. In order to separate the contribution of H_2O_2 from that of the radicals OH and O_2H in the enzyme inhibition, 1 microgram of catalase was added to the test

TABLE III

Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase

Enzyme activity determined by the value of $K = \frac{1}{t} \times \frac{C_0 - C}{C_0 C}$ where t is time in minutes; C_0 , initial concentration of DPN (2.5×10^{-7} M); C , concentration of DPN at time t .

Irradiation		K values		Inhibition
days	r	Control	Polonium	per cent
		$\times 10^5$	$\times 10^5$	
1	180	10	4.15	58
2	360	10	2.7	73
4	720	10	0.8	92
6	1080	10	0	Complete

TABLE IV

*Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase
Reactivation with Glutathione*

The control K values were those obtained after addition of glutathione.

Irradiation 0.57 microcuries per cc.	Inhibition by α rays	K values		Inhibition
		After glutathione		
		Control	Polonium	
<i>days</i>	<i>per cent</i>	$\times 10^5$	$\times 10^5$	<i>per cent</i>
1	58	14.6	8.3	43
2	73	13.5	6.8	50
4	92	13.5	4.7	65
6	Complete	11.8	3.8	68

tubes previous to the addition of polonium. Catalase protected the enzyme partially from the inhibitory action of alpha rays, the protection being from 41 to 56 per cent of the total inhibition (Table V). This protective action of catalase is due to the destruction of H_2O_2 formed on irradiation and not to the protection reported by Dale (9), because crystalline egg albumin added at a molar concentration 1,000 times greater than that of catalase (18 micrograms) had no effect at all.

Effect of Beta Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase.—Beta rays acted as powerful inhibitors of the enzyme, for inhibition was

observed even after irradiation with 14 r (1 day) (Table VI). However, re-activation of the enzyme was not obtained on addition of glutathione.

TABLE V
*Effect of Alpha Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase
Protection with Catalase*

Irradiation 0.57 microcuries per cc.	Inhibition by α rays	K values		Inhibition
		Catalase addition		
		Control	Polonium	
days	per cent	$\times 10^5$	$\times 10^5$	per cent
1	58	10.7	7.6	24
2	73	11.5	6.8	32
4	92	10.4	4.7	53
6	Complete	10	3.5	65

TABLE VI
Effect of Beta Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase

Irradiation 0.42 microcuries per cc.		K values		Inhibition
		Control	β rays	
days	r	$\times 10^5$	$\times 10^5$	per cent
1	14.6	10	9	10
2	29	10	8.3	17
4	56.5	10	6.8	32
6	88	10	6.3	37

TABLE VII
*Effect of Beta Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase
Protection with Catalase*

Phosphoglyceraldehyde dehydrogenase, 140 micrograms; catalase, 1 microgram. Sr^{90} Cl_2 0.888 microcuries. Volume, 2.1 cc.

Irradiation	Inhibition	K with catalase		Inhibition
		Control	β ray	
days	per cent	$\times 10^5$	$\times 10^5$	per cent
1	10	10.7	10	6.5
2	17	11.5		
4	32	10.4	10	4
6	37	10	9.5	5

Previous addition of catalase protected the enzyme effectively, especially after prolonged irradiation (Table VII). No explanation can be offered for the lack of enzyme reactivation.

Effect of Gamma Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase.—In these experiments a very dilute solution of enzyme was used, five times less than in the previous experiments. Half-inhibition was produced on irradiation with 50 r (Table VIII). Addition of glutathione after irradiation produced no reactivation.

Effect of Gamma Rays on the Activity of Urease.—For the irradiation of urease with gamma rays the experiments were performed at first in the presence of glycine (0.1 M), because addition of glycine allowed more quantitative deter-

TABLE VIII

Effect of Gamma Rays on the Activity of Phosphoglyceraldehyde Dehydrogenase
Amount of enzyme 14 micrograms. Buffer, phosphate 0.02 M; pH, 7.

Dose	K value	Inhibition
r	$\times 10^5$	per cent
None	4.2	
25	3.5	17
50	2.0	54
200	1.4	67

TABLE IX

Inhibition of Urease by Gamma Ray Irradiation
Protection with Glycine

Urease, 1.7 micrograms in 1.1 cc. phosphate buffer, pH 7. Enzyme activity given in units. Unit as defined by Sumner and Hand (18).

Dose	Glycine	Control	Gamma rays	Inhibition
r	M	units	units	per cent
100		1,000	760	24
200		1,000	705	30
100	10^{-1}	1,190	1,196	None
100	10^{-2}	1,190	1,159	"
100	10^{-4}	1,190	1,137	4.5
100	10^{-6}	1,190	1,079	9

minations of enzyme activity. It was found, however, that glycine protected the enzyme from inhibition. A concentration of 10^{-2} M protected it completely, while in the absence of glycine 100 r produced an inhibition of 24 per cent. Even 10^{-6} M glycine protected 9 per cent (Table IX).

The mechanism of this protective action is not known. It must be recalled that Bailey (10) found that the enzyme activity of adenosinetriphosphatase (another sulphydryl enzyme) was considerably enhanced on addition of glycine and other amino acids. Glycine is known to form complex salts with heavy metals.

Enzyme inhibition by gamma rays was not released on addition of glutathione. This lack of reactivation was taken advantage of to demonstrate definitely that inhibition of the enzyme by gamma rays is due to oxidation of the sulfhydryl groups of the protein. Hellerman *et al.* (3) showed that *p*-chloromercuribenzoate inhibits urease by combination with the —SH groups (formation of the compound R-S-Hg-benzoate), and that inhibition is released on addition of a sulfhydryl-containing substance. If enzyme inhibition by gamma rays were due only to oxidation of the —SH groups by the oxidizing products of irradiated water, there would be no inhibition on irradiation of urease when the —SH groups were protected by *p*-chloromercuribenzoate. If inhibition were due to destruction or denaturation of the enzyme, it would occur even after conversion of the —SH groups to the R-S-Hg-benzoate. Urease was

TABLE X
Inhibition of Urease by Gamma Ray Irradiation
Protection with p-Chloromercuribenzoate

Urease, 1.7 micrograms in 1.1 cc. *p*-Cl-Hg-benzoate (*p*-Cl-Hg), 0.0001 M; glutathione (GSH), 0.01 M.

Dose	Enzyme	Enzyme + <i>p</i> -Cl-Hg	Enzyme + GSH	Enzyme + Hg + GSH
<i>r</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>
0	1,492	0	1,538	1,498
200	989	0	939	1,466

irradiated with 200 r of gamma rays in the presence of glutathione (0.001 M) and in the presence of *p*-Cl-Hg-benzoate (0.0001 M). Urease with glutathione was inhibited to the same extent as the enzyme alone. When to the irradiated enzyme containing *p*-Cl-Hg-benzoate there was added glutathione, the enzyme activity was restored completely (Table X). Protection of the —SH groups by formation of the reversible mercaptide compound protected the enzyme from the inhibiting action of gamma rays.

The Ionic Yields of Enzyme Inhibition by Ionizing Radiations.—There is little information on the relative efficiency of different radiations regarding chemical effects. In reactions in the gaseous state, the ionic yields with different ionizing radiations are in general similar. In the production of H₂O₂ on irradiation of oxygenated water, Frilley (8) reports similar ionic yields for x-ray and for alpha ray irradiation. Lanning and Lind (11) found that fairly strong solutions of HBr, HI, and KMnO₄ were decomposed by alpha rays with an ionic efficiency of the order of unity. Irradiation of tyrosine by alpha rays seems, however, far less efficient than by x-rays, according to Nurnberger (12). On irradiation of carboxypeptidase with alpha rays (irradiation with radon) and

with x-rays, Dale, Meredith, and Gray² found that the efficiency of alpha rays was only 5 to 9 per cent that of x-rays. Numerous biological effects have been measured simultaneously with x-rays and with gamma rays, such as the inhibition of mitosis in tissue cultures (13), the lethal action on *Drosophila* eggs (14), on *Drosophila* pupa (15), on mouse tumors (16). In all cases, the efficiency of gamma rays was 50 to 20 per cent less than that of x-rays.

Calculation of the ionic efficiency of alpha, beta, and x-rays on inhibition of crystalline phosphoglyceraldehyde dehydrogenase has shown that all three ionizing radiations had about the same efficiency, namely unity (Table XI). With gamma rays, the ionic yield was 0.7.

TABLE XI

Ionic Yields of Enzyme Inhibition by X-, Alpha, Beta, and Gamma Rays

Enzyme: Phosphoglyceraldehyde dehydrogenase (70 micrograms per cc., except in gamma rays where 14 micrograms were used). *M*, number of enzyme molecules inhibited; *N*, number of ion pairs produced on ionization of 1 cc. of water, assuming that x-rays produce 1.616×10^{12} ; alpha rays, 1.90×10^{12} ; beta rays, 1.8×10^{12} ; and gamma rays, 1.79×10^{12} .

Ionizing radiation	Dose <i>r</i>	<i>M</i>	<i>N</i>	Ionic yield
X-rays	200	3.01×10^{14}	3.23×10^{14}	0.93
Alpha rays	180	3.49×10^{14}	3.42×10^{14}	1.0
Beta rays	56.5	1.93×10^{14}	1.02×10^{14}	1.9
Gamma rays	50	6.51×10^{13}	9.5×10^{13}	0.7

DISCUSSION

The experiments presented here on the inhibition of the sulfhydryl enzymes, phosphoglyceraldehyde dehydrogenase and urease, by alpha, beta, and gamma rays, and reactivation (in the case of alpha rays) on addition of glutathione, are presented as further evidence that ionizing radiations inhibit sulfhydryl enzymes by oxidation of the —SH groups essential for enzyme activity. This specific action on the —SH groups was clearly shown in the urease experiments and irradiation with gamma rays. A dose of gamma radiation that inhibited the enzyme containing the —SH groups intact had no effect at all when the —SH groups were withdrawn from oxidation by their transformation into mercaptides. In fact, complete reactivation of the enzyme was obtained on addition of glutathione.

The rôle of H_2O_2 in the inhibition of sulfhydryl enzymes by ionizing radiations was shown by the partial protection produced on addition of small amounts of

² Dale, W. M., Meredith, W. J., and Gray, L. H., The inactivation of an enzyme (carboxypeptidase) by x- and α radiation. Manuscript kindly sent to one of us by Dr. Gray.

catalase. This inhibiting action of H_2O_2 is probably restricted to oxidation of —SH groups. On irradiation with alpha rays, oxidation by H_2O_2 contributed 30 per cent of the total inhibition, while with beta rays there seemed to be a greater contribution.

The equal efficiency of alpha rays and x-rays in the inhibition of sulfhydryl enzymes, as contrasted with the greatly diminished efficiency of alpha rays in the inhibition of carboxypeptidase, is probably due to the different mechanisms of action. The former are inhibited by oxidation of the —SH groups, while carboxypeptidase inhibition seems to be due to protein denaturation (the mechanism of carboxypeptidase action is unknown). All ionizing radiations had the same efficiency in inhibiting phosphoglyceraldehyde dehydrogenase.

Ionizing radiations have two different actions on proteins: oxidation of their —SH groups—a reversible phenomenon—and denaturation and destruction of the molecule, an irreversible phenomenon. The first requires fewer ionizing radiations than the second. These observations become of considerable biological significance when they are considered together with the distribution of sulfhydryl groups in living cells. In fact, it has been shown by a number of investigators (see Brachet (17)) that an abundance of sulfhydryl compounds are required by cells in mitosis and in division and growth. In all probability, these sulfhydryl groups (which are different from the sulfhydryl groups of enzymes) are oxidized on irradiation of cells, and inhibition of mitosis and of cell division by ionizing radiations may be due to this oxidation. Since oxidation of sulfhydryl groups is in general a reversible process, the effects of small amounts of ionizing radiations might also be reversible.

SUMMARY

The activity of crystalline phosphoglyceraldehyde dehydrogenase and urease was decreased when dilute solutions of these sulfhydryl enzymes were irradiated with small doses of alpha rays from Po, beta rays from Sr^{89} , and gamma rays from Ra. Partial reactivation of the enzyme by addition of glutathione was obtained after inhibition with alpha rays. Evidence that these inhibitions are due to oxidation of the —SH groups of the enzymes was given by the irradiation of the mercury-mercaptide urease with gamma rays. This irradiated complex was completely reactivated by glutathione as was the non-irradiated enzyme. The ionic efficiency of all these ionizing radiations on inhibition of phosphoglyceraldehyde dehydrogenase was similar (ionic yield around 1).

The sulfhydryl groups of crystalline phosphoglyceraldehyde dehydrogenase were titrated by enzyme activity measurements and by ferricyanide oxidation.

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PEPTIDASE ACTIVITIES OF EXTRACTS OF SALIVARY GLANDS OF *DROSOPHILA MELANOGASTER**†

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Analysis of cell growth in chemical terms is essentially an analysis of the formation and activity of enzymes. The enzymes must first be characterized as a basis for the measurement of their activities during the growth of suitable biological material. The work reported here served this function preparatory to a subsequent study of changes in enzyme content during growth of the larval salivary gland of *Drosophila melanogaster*. The peptidases were selected for studies of growth because their specificities and their distribution in tissues have suggested a correlation with protein synthesis (Linderstrøm-Lang and Holter, 1932; Fruton, 1941; Bergmann, 1942). Discussion of the problems raised by this possibility and of the choice of biological material will be deferred until the data on growth are presented (Patterson, Dackerman, and Schultz, 1949).

Since the peptidases of *Drosophila* had not previously been studied, it was first necessary to ascertain whether the larval salivary gland contained a sufficient quantity of recognizable enzymes to make its use feasible in experimental work. The titrimetric methods of Linderstrøm-Lang and Holter (1940) proved suitable for determination of peptidase activity on individual salivary glands or extracts from them.

Proteolytic enzymes are classified into exo- and endopeptidases according to the type of simple peptide substrate attacked (Bergmann, 1942). Five substrates for determination of exopeptidase activity and one suitable for identification of a type of endopeptidase activity were available. Crude glycerine extracts of salivary glands were analyzed for peptidase activity toward these different substrates under various conditions of activation and inhibition. The data were then compared with those obtained from partially purified enzyme preparations by other workers, and were interpreted in terms of specific peptidases in the extracts. This rough survey proved sufficient to demonstrate the presence of one enzyme with a high enough activity to make practicable its use for the study of changes during growth. This enzyme, a peptidase splitting alanyl-glycine, was further studied in the extracts not only to assure its identity

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with the usual alanyl-glycine peptidase, but also to find the best conditions for its measurement. Accordingly, the behavior of the enzyme towards activators and inhibitors was investigated in greater detail. In addition, its pH activity, time, and concentration curves were determined.

Altogether, the results have shown that both in the variety of enzymes and in the ease with which their activity can be measured by micro methods, the *Drosophila* salivary glands are favorable material.

EXPERIMENTAL PROCEDURES

Biological Material

Of the three stocks of *Drosophila melanogaster* used in these experiments two were wild type: Tuscaloosa (Tusc) and the highly inbred Oregon R (Ore R). The third, a giant mutant, was chosen to provide individuals with large salivary glands. This stock (gt w^a Ore R) was isogenic with Ore R, except for the giant mutant and an apricot allele at the white locus.

Salivary glands were taken from individuals at three stages of development: (a) late third instar larvae (LL) that had crawled up the sides of the bottle and were no longer feeding; (b) prepupae (5), time at 5 hours (25°C.) after the larva with the pupal horns everted was immobile; and (c) everted pupae (ev); i.e., approximately 12 hour pupae in which eversion of the imaginal discs (pupation proper) has just occurred. In the first two stages, the larval glands were still growing; at the later stage they were beginning to disintegrate, although still grossly whole in appearance.

Dissection

In view of the instability of the peptidases, precautions were taken to minimize inactivation during dissection of the glands out of the larva. The larvae and pupae were rinsed in fly-Ringer's solution (Ephrussi and Beadle, 1936) and any adherent food was removed before they were ice-cooled. They were then dissected in this Ringer's solution on a cold stage which consisted of a specially designed insulated ice chamber fitted into the stage of a binocular dissecting microscope. The glands were removed by the usual method of decapitation of the larva, or by cutting off the tip of the pupa.

The next step, the dissection of the fat body from the glands, was the most time-consuming and painstaking part of the experiment. The fat body is closely adherent to the late larval glands, is easily removed from the 5 hour pupal glands, and is rarely present on the everted pupal glands. Extracts of the fat body taken out of the same larvae from which the glands had been dissected, showed considerable peptidase activity (see Table IV). It was therefore necessary to dissect the fat away with care. This operation, the removal of the ducts and the ring of imaginal cells at the junction of duct and gland, and the transfer of the glands to a fresh drop of Ringer's, were all accomplished with steel needles. Smooth tipped glass needles were used for the rapid transfer of the glands, one by one, from the Ringer's through a rinsing drop of glycine buffer into the final medium.

Preparation of Extracts

The extraction medium used for the exopeptidase experiments was 30 per cent glycerine buffered to pH 7.4 with $m/60$ phosphate. Without glycerine to stabilize the enzyme, the activity of extracts in splitting alanyl-glycine is rapidly lost (Linderstrøm-Lang, 1929, 1930). A standard extract¹ consisted of 36 glands added successively to 234 (extracts 1 to 15) or 305 μ l. (extracts 16 to 26 and 29) of glycerine buffer in a 1 ml. centrifuge tube with ground glass stopper. All volumes under 1 ml. were pipetted into the tubes by the Levy (1936) type constriction pipettes.

For the endopeptidase experiments, the extraction medium was 30 per cent glycerine buffered with $m/10$ citrate to pH 5. Two concentrated extracts were prepared, each containing 108 glands in 234 μ l. of buffer.

Great care was taken to ensure both clean and sterile conditions in the extracts and the reaction tubes. Dust contamination reduced the enzymatic activity and bacterial contamination added enzymes other than those measured. Therefore, after the earliest experiments, all work was carried out in an air-conditioned room, rendered dust-proof as far as possible. Finally, as the extracts were prepared, they were sterilized by freezing and thawing seven times. Dry ice-acetone mixtures were used for the quick freezing. Routine cultures of extracts, buffers, and substrate solutions were made. The solutions rarely showed contamination and the data from experiments using contaminated solutions are omitted.

During extraction the glands underwent no chemical or physical treatment other than freezing and thawing. The use of whole cells was advocated by Linderstrøm-Lang (1933) in order to preserve the enzyme more nearly in its native state than it would be in cells exposed to mechanical handling or autolysis. Rapid freezing and thawing neither changed the enzymatic activity nor cytolysed the cells of the *Drosophila* salivary gland. When other usual cytolytic procedures were tried a lowered enzymatic activity resulted, hence no bound enzyme could be freed in this way. With this material, therefore, the use of whole glands in which the cells were not cytolysed seemed advisable, especially since no plans had been made at this time to study fragments of the glands or cells.

During the dissection period the extract tubes remained at room temperature (23–24°C.); thereafter extraction was allowed to proceed at 4°C. Under these conditions the peptidase activity of the extracts was found to increase for about 13 days, remaining constant after that time. Extracts were generally used in the period between 13 and 23 days' extraction, but comparisons were made only between extracts of comparable extraction periods. A check on the completeness of extraction of the enzyme was carried out by reextracting the glands used for the extracts. No enzyme activity was detected in these second extracts.

The Measurement of Peptide Hydrolysis

The substrates used for the exopeptidase experiments included *DL*-alanyl-glycine (AG), *DL*-leucyl-glycine (LG), *L*-leucylglycyl-glycine (LGG), glycylglycyl-glycine (GGG) and glycyl-glycine (GG). The racemic mixtures were made up to be 0.18 M and the

¹ We wish to thank Miss Dorothy Newmeyer for help in preparing extracts 1 to 19.

l-forms 0.09 M. They were adjusted to a given pH at 25°C. with 0.1 N NaOH using a Beckman pH meter. Since the experiments were carried out at 40°C., the pH readings at 25°C. had to be converted to the correct values for the higher temperature, making allowance for the change in *pK* of the peptide with temperature (Cohn and Edsall, 1943). Actual readings at 40°C. with the pH meter showed that the corrections were accurate for AG. In all but the pH activity experiments, a pH of 7.60 ± 0.05 at 40°C. was used. This corresponds to a pH of 7.95 ± 0.05 at 25°C., in the case of AG. This pH was close to the optimum found for the splitting of AG and was considered near enough to the published optima for enzymes splitting the other substrates to be used for these survey experiments. The buffering capacity of the substrate was sufficient so that during the course of an experiment no measurable change in pH occurred, even at maximal observed splitting (8 μ l. N/20 HCl).

α -*N*-Benzoyl-*l*-arginineamide² (BAA) served as substrate for the endopeptidase work (Fruton, Irving, and Bergmann, 1941). The BAA was made up to give a final concentration of 0.05 M. Here 30 per cent glycerine buffered to pH 5.00 with citrate (0.04 M final concentration) was used as the extraction medium. Cysteine (0.01 M) was employed for activation.

The hydrolysis was carried out according to the methods of Linderstrøm-Lang and Holter (1940); (see also Linderstrøm-Lang, 1938). Small reaction tubes were used to which were added a 7 μ l. drop of extract or buffer and a 7 μ l. drop of substrate solution. All experiments were run in a water bath at 40.0°C. and the temperature was maintained constant to $\pm 0.03^\circ\text{C}$. When AG or LG served as substrate, a reaction time of 4 hours sufficed. The other substrates required a longer period of hydrolysis (20 to 24 hours).

The reaction tubes were set up at 6 minute intervals, and after having been capped, remained in the bath for the allotted time. Reactions were stopped, maintaining the requisite 6 minute intervals, by the addition of 30 μ l. of titration fluid (N/20 HCl in 97 per cent alcohol) from an automatic pipette. About 150 μ l. of indicator (*ca.* 0.001 per cent naphthyl red in 90 per cent acetone) were added and the titration of the amino groups was carried out immediately with the aid of a contact type burette,³ which holds 100 μ l. and can be read to 0.02 μ l. This procedure allowed no time for evaporation of the titration fluid with which the reaction was stopped, thus eliminating a possible source of error.

Three sets of tubes were used in each experiment: "determinations," "enzyme blanks," and "reagent blanks." All were titrated to a standard pH at the end of a given reaction time. A "determination" consisted of a 7 μ l. aliquot of enzyme extract to which was added a 7 μ l. drop of substrate solution, the two drops being mixed with the aid of a stirring bead (flea) and magnet. The "enzyme blanks" consisted likewise of a drop of enzyme extract, but in this case the substrate drop was placed on the side

² The triglycine and benzoyl-*l*-arginineamide were kindly supplied by Dr. Jesse Greenstein of the National Cancer Institute, Bethesda. We have to thank Dr. Gerrit Toennies of the Institute for Cancer Research for the rest of the substrates, which were Hoffmann-La Roche peptides.

³ We wish to thank Dr. W. L. Doyle of the University of Chicago for the use of one of his burettes in the early part of the work.

of the tube. These tubes were suspended in the water bath in a horizontal position so that there would be no contact between enzyme and substrate. The "reagent blanks" consisted of a drop of glycerine buffer mixed with a drop of substrate.

The difference between the titration values of determination and enzyme blank was used as a measure of the hydrolysis of the substrate caused by the enzyme. For simplicity, all enzyme contents are recorded in terms of this titration value. The values recorded in the tables and graphs represent the mean of three determinations minus the mean of two or three enzyme blanks. When the average deviation is given followed by a +, this means that one of the enzyme blanks was lost. Average deviation is used here rather than standard deviation since the numbers are very small. The difference between the titration values of the enzyme blank and the reagent blank gave the "enzyme blank value," *i.e.* the value due to any free amino acid or other titrable acid or alkali originally present in the glands or extract, or due to autolysis. This value was small, averaging 0.20 μ l. in the extracts buffered at pH 7.4. In the extracts prepared for determination of endopeptidase activity, however, it was very high, rising with the time of extraction. These extracts were buffered to pH 5.0.

When experiments were carried out to test the effect of added substances, a small drop (usually 1 μ l.) of the solution in question was added to the 7 μ l. drop of enzyme extract or buffer. The concentration of added substance given in the tables is that of the final mixture after substrate addition. The drops were thoroughly mixed and the tube incubated in the water bath for one-half hour before addition of the substrate drop. Control experiments were carried out in which the extract and buffer drops were diluted with a similar volume of water.

RESULTS

Exopeptidase Activity of Extracts

The activity of gland extracts in hydrolyzing various peptide substrates is given in Table I. The hydrolysis of AG is at least twice as great as that of LG whether or not the latter is activated by MgSO_4 . Glycylglycine shows very little splitting even with a reaction time of 20 hours. The hydrolysis of the tripeptides LGG and GGG by gland extracts is low, but it is measurable in the case of LGG in 20 hours. The splitting of GGG is always very low.

It will be evident from an inspection of the data that the activities in the extracts from male glands are consistently lower than those from female glands. For the AG hydrolysis, this will be discussed when data from single gland experiments are considered (Patterson, Dackerman and Schultz, 1949). Further data are required to determine how the activities toward the other substrates vary in the extracts of glands from the two sexes. The present experiments indicate that differences of this kind may exist.

As seen from the average deviations, the variability in the case of some of the determinations is great. Since the average deviation in the reagent blanks containing glycerine buffer and alanylglycine was only about ± 0.06 μ l. N/20 HCl the variability was probably caused by difficulty in pipetting aliquots of

TABLE I
Hydrolysis of Peptide Substrates by Extracts of Salivary Glands
(pH 7.60 \pm 0.05, 40°C.)

Extract No.	Stock	Stage	Sex	Time extracted	Substrate	Time reaction	MnSO ₄ concentration	Hydrolysis*
				days		hrs.	M	$\mu\text{L N}/20 \text{ HCl}$
14	Tusc	ev	♀	22	LG	4	0.003	2.21 \pm 0.16
				27	LGG	4	0.003	0.38 \pm 0.10
				27	GGG	4	—	0.38 \pm 0.04
				28	LG	4	0.003	1.83 \pm 0.01 +
15	Tusc	ev	♂	25	LG	4	0.003	1.48 \pm 0.35
				28	LG	4	0.003	1.69 \pm 0.06
				28	LGG	20	0.003	1.96 \pm 0.13
				28	GGG	20	—	0.33 \pm 0.23
16	Tusc	ev	♀	16	LG	4	0.003	2.02 \pm 0.27
				22	LGG	20	0.003	3.25 \pm 0.13
				22	GGG	20	—	0.37 \pm 0.17
				26	AG	4	—	4.39 \pm 0.22
17	Tusc	ev	♂	16	LG	4	0.003	1.27 \pm 0.27
				20	LGG	20	0.003	1.66 \pm 0.26
				20	GGG	20	—	0.43 \pm 0.14
				26	AG	4	—	2.72 \pm 0.08
18	Tusc	LL	♀	17	LG	4	0.003	1.64 \pm 0.04
				21	LGG	20	0.003	1.97 \pm 0.30
				21	GGG	20	—	0.47 \pm 0.08 +
				24	AG	4	—	3.84 \pm 0.21
19	Tusc	LL	♂	18	LG	4	0.003	1.16 \pm 0.06
				20	LGG	20	0.003	1.16 \pm 0.36
				20	GGG	20	—	0.94 \pm 0.10
				24	AG	4	—	3.35 \pm 0.04
20	Ore R	5	♀	13	AG	4	—	4.57 \pm 0.39
				24†	LG	4	0.001	1.94 \pm 0.40 +
22	Ore R	5	♀	21‡	AG	4		3.37 \pm 0.04 +
				21‡	GG	4		0.24 \pm 0.26
				21‡	GG	20		0.87 \pm 0.32
24§	Ore R	5	♀	16	AG	4		6.21 \pm 0.36
				20	LG	4		2.60 \pm 0.12
26	gt w*	5	♀	15	AG	4		5.28 \pm 0.43
	Ore R			15	LG	4		2.36 \pm 0.31

* Titration value given by a 7 μL aliquot of gland extract. These values may be converted to splitting per gland by multiplying by the factor 0.89 in extracts 1 to 15; 1.16 in extracts 16 to 23; 1.10 in extracts 24, 25, and 29, and 2.32 in extract 26.

† Frozen at -70°C . after 14 days.

§ 38 glands added.

|| Extract diluted 1:1 with glycerine buffer.

the highly viscous gland extracts. This viscosity, together with the ultraviolet absorption spectrum of the extracts (Fig. 1) indicated that they contained considerable amounts of nucleic acid. Calculations based on the phosphorus content and the absorption at 2600 Å of a solution of yeast nucleic acid (pH 6.8),

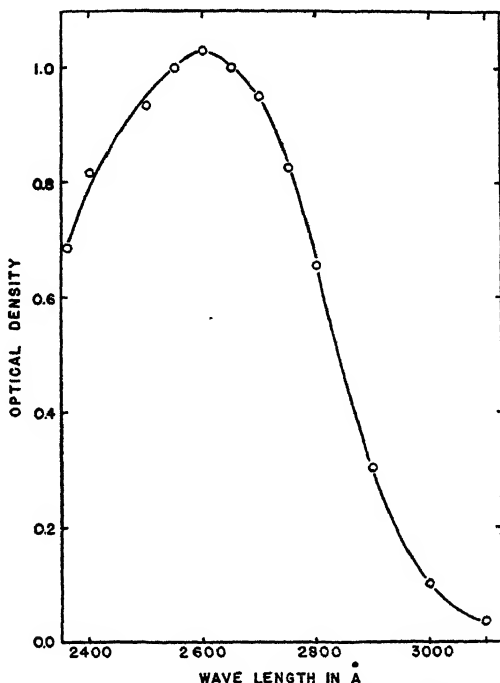


FIG. 1. Ultraviolet absorption spectrum of a buffered (M/60 phosphate, pH 7.4) 30 per cent glycerine extract of salivary glands from gt w^a Ore R 5 hour female pupae. After 14 days at 4°C. the extract (36 glands in 305 μ l. buffer) was diluted 1:1 with glycerine buffer and the absorption measured against a glycerine buffer blank in a Beckman spectrophotometer. Micro cells (Lowry and Bessey, 1946) containing 60 μ l. drops were used.

purchased from Schwarz Laboratories, gave a concentration of 0.05 mg. per ml. for five of the standard gland extracts.⁴

Endopeptidase Activity: Benzoyl-L-Arginineamide Hydrolysis

The endopeptidase experiments with BAA as substrate serve to indicate the presence of cathepsin II (Fruton, Irving, and Bergmann, 1941) in pH 5.0 glycerine buffer extracts of both males and females (ev). Without cysteine activa-

⁴ *Note Added in Proof.*—Analysis by the orcinol reaction has given values of the same order of magnitude, showing the nucleic acid to be of the pentose type. Tests with the diphenylamine reaction have proved negative.

tion, there was no activity towards BAA; but with the activator, a low hydrolysis was observed, for example, $0.98 \pm 0.14 \mu\text{l. N/20 HCl}$ in 24 hours at 40°C . This value should perhaps be considered a minimal measure of enzyme content, since its high enzyme blank (4.37 ± 0.14) shows the presence of split products. This high autolysis, after a 21 day extraction period at pH 5.0, 4°C , obviously indicates the presence of other enzymes, possibly other peptidases. Further experiments are necessary at diverse pH using short extraction periods and a variety of substrates.

Activity of Extracts in the Presence of Added Substances

The differences in the activity of the extracts toward diverse substrates indicated that there were probably several enzymes present. Since AG, LG, and LGG were the substrates showing the greatest and therefore the most accurately measurable splitting, only the enzymes concerned in their hydrolysis were considered. These enzymes might not be present in fully activated form in the extracts and it was therefore necessary to carry out a series of experiments to test the effects of addition of various substances known as activators of peptidases.

It has become increasingly clear recently that the dipeptidases and aminopeptidases are metal enzymes (Maschmann, 1943; Smith, 1946, 1948 b). Manganese or magnesium, zinc, and cobalt are the metals whose action as coenzymes has been demonstrated. The peptidase for which the greatest volume of data exists is *l*-leucineaminopeptidase (Berger and Johnson, 1939, 1940; Smith and Bergmann, 1944). This enzyme splits LG and LGG at equal rates and is activated primarily by manganese and to a lesser degree by magnesium. When the gland extracts were incubated with MnSO_4 (0.003 M) for one half-hour before substrate was added, it was found (Table II) that the splitting of LG was not activated whereas the much slower hydrolysis of LGG was enhanced by this metal. Since Smith (1946) has shown the metal-protein combination to be a slow reaction, a 4 hour preincubation of the extract with Mn^{++} was tried in an attempt to activate the LG-splitting enzyme. No appreciable increase in hydrolysis was observed. These results are discussed in a later section as evidence for the presence of two distinct enzymes whose activity is measured here by the splitting of LG and LGG.

Since activation experiments did not succeed in bringing the value for the hydrolysis of LG up to those shown by the gland extracts when AG was used as a substrate, AG was obviously the most convenient substrate to use. The next step was to find out whether the splitting of AG could be activated. As seen from Table III when Mn^{++} , Zn^{++} , or Co^{++} were added in the concentrations commonly found to give activation, no clear-cut enhancement or inhibition of splitting was observed. From these experiments it follows that whatever the relation between enzyme and metal, no gain in activity results from the addition of these metals to the extracts when the substrate AG is used. If

sufficient metal is present in the extracts, addition of more might produce only an inhibition.

TABLE II

Effect of Manganese on the Hydrolysis of Leucylglycine and Leucylglycylglycine by Extracts of Salivary Glands of Drosophila melanogaster
(pH 7.60 \pm 0.05, 40°C.)

Extract No.	Stock	Stage	Sex	Time ex- tracted	Substrate	Time reaction	MnSO ₄ concentra- tion	Time act	Hydrolysis*
				days		hrs.	M	hrs.	$\mu\text{L.N/20 HCl}$
14	Tusc	ev	♀	22	LG	4	0.003	0.5	2.21 \pm 0.16
				22	LG	4	—	0.5	2.01 \pm 0.10
				27	LGG	4	0.003	0.5	0.38 \pm 0.04
				27	LGG	4	—	0.5	0.00
15	Tusc	ev	♂	25	LG	4	0.003	0.5	1.48 \pm 0.35
				25	LG	4	—	0.5	0.72 \pm 0.30
				28	LGG	20	0.003	0.5	1.96 \pm 0.13
				28	LGG	20	—	0.5	0.90 \pm 0.37
16	Tusc	ev	♀	16	LG	4	0.003	0.5	2.02 \pm 0.27
				16	LG	4	—	0.5	1.97 \pm 0.17
				22	LGG	20	0.003	0.5	3.25 \pm 0.13
				22	LGG	20	—	0.5	1.07 \pm 0.28
17	Tusc	ev	♂	16	LG	4	0.003	0.5	1.29 \pm 0.27
				16	LG	4	—	0.5	1.52 \pm 0.11
				20	LGG	20	0.003	0.5	1.66 \pm 0.26
				20	LGG	20	—	0.5	0.64 \pm 0.07
18	Tusc	LL	♀	19	LG	4	0.003	0.5	1.64 \pm 0.04 +
				19	LG	4	—	0.5	1.86 \pm 0.06 +
				21	LGG	20	0.003	0.5	1.97 \pm 0.30
				21	LGG	20	—	0.5	1.47 \pm 0.35
19	Tusc	LL	♂	18	LG	4	0.003	0.5	1.16 \pm 0.06
				18	LG	4	—	0.5	1.90 \pm 0.16 +
				20	LGG	20	0.003	0.5	1.16 \pm 0.36
				20	LGG	20	—	0.5	0.67 \pm 0.08 +
20	Ore R	5	♀	24†	LG	4	0.001	4	1.94 \pm 0.40 +
				24†	LG	4	—	4	1.45 \pm 0.04 +

Notes as under Table I.

It seemed desirable to determine whether the enzymes of these extracts showed a conventional behavior toward known inhibitors of peptidases (Grassmann and Dyckerhoff, 1928; Gailey and Johnson, 1941). In Table IV experiments with cysteine (SH inhibition) are given and the resulting inhibition com-

TABLE III

Effect of Metals on the Hydrolysis of Alanylglycine by Extracts of Salivary Glands of Drosophila melanogaster

(pH 7.60 \pm 0.05, 40°C., 4 hours)

Extract No.	Stock	Stage	Sex	Time extracted	Metal salt	Concentration	Hydrolysis*
				days			
18	Tusc	LL	♀	24	MnSO ₄ —	0.003	4.04 ± 0.47 3.84 ± 0.21
19	Tusc	LL	♂	24	MnSO ₄ —	0.003	2.42 ± 0.19 $3.35 \pm 0.04 +$
23	Ore R	5	♂	23‡	ZnSO ₄ —	0.0004	1.53 ± 0.65 0.82 ± 0.34
25§	Ore R	5	♂	17	ZnSO ₄ —	0.0004	3.01 ± 0.52 4.51 ± 0.19
29§	Ore R	5	♀	8	CoCl ₂ —	0.001	1.40 ± 0.34 2.37 ± 0.32

Notes as under Table I.

TABLE IV

Effect of Inhibitors on the Hydrolysis of Peptides by Extracts of Salivary Glands and Fat Body of Drosophila melanogaster

(pH 7.60 \pm 0.05, 40°C., 4 hours)

Extract No.	Stock	Material	Sex	Time extracted	Substrate	Inhibitor	Concentration	Hydrolysis*	Inhibition
				days					per cent
7	Tusc	Fat body	♀	28	AG	Cysteine —	0.01	0.84 ± 0.40 $8.07 \pm 0.15 +$	90
6	Tusc	Fat body	♂	27	AG	Cysteine —	0.01	$0.51 \pm 0.11 +$ 7.13 ± 0.15	93
23	Ore R	5	♂	20‡	AG	Cysteine —	0.01	0.00 1.90 ± 0.21	100
25	Ore R	5	♂	23	AG	Cysteine —	0.001	2.08 ± 0.12 3.57 ± 0.06	40
24§	Ore R	5	♀	20	LG	Cysteine Cysteine —	0.01 0.001	1.07 ± 0.47 2.38 ± 0.44 2.60 ± 0.12	(59)¶ (15)
25§	Ore R	5	♂	17	AG	Alanine —	0.01	4.27 ± 0.13 4.51 ± 0.19	(5)

Notes as under Table I.

¶ Values in parentheses only qualitatively significant.

pared with that by another amino acid, alanine. AG splitting was inhibited 90 to 100 per cent by cysteine at 0.01 M and about 40 per cent at 0.001 M. The hydrolysis of LG was also inhibited but to a lesser degree, again giving evidence for the possible existence of separate enzymes of different specificity. When AG was used as substrate, alanine at 0.01 M gave only the expected slight inhibitory effect of a split product.

The experiments in this section show that the extracts of salivary glands on the whole behave in a manner similar to preparations of peptidases from other sources. They also provide some justification for using the splitting of AG

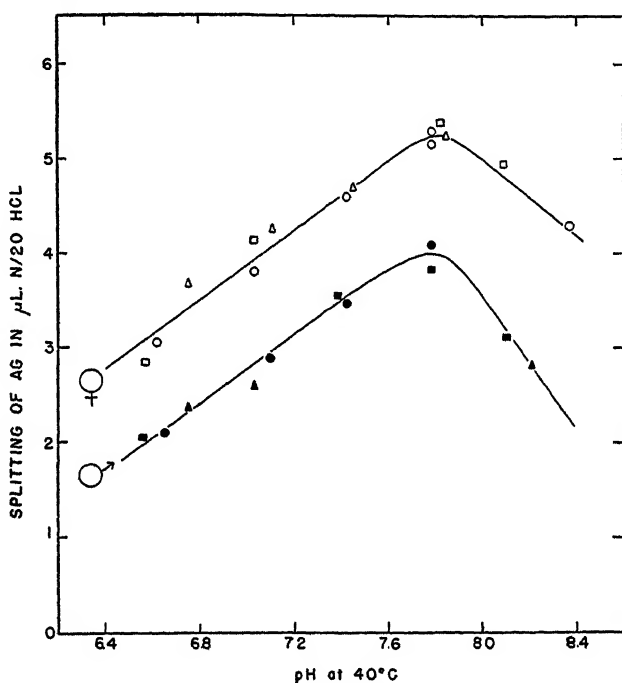


FIG. 2. pH activity curve of the enzyme-splitting AG in extracts of salivary glands from the Tusc stock. The values were taken from different extracts. The solid symbols represent extracts from males prepared simultaneously with those from females (open symbols); \circ , \triangle extracts from late larvae, and \square from pupae.

without addition of metal activators as an index of the peptidase content of the salivary glands.

Determination of Optimal Conditions for Measurement of AG Peptidase

In the preceding brief survey of the effects of activators, etc., some of the conditions for the use of measurement of AG hydrolysis as an index of enzyme content have been established. In the present section, other necessary data are presented: measurement of the activity of the enzyme extracts at a series of different pH, concentrations, and reaction times.

The pH activity curves (Fig. 2) were constructed from determinations made on a series of comparable extracts, the substrate adjusted to the required pH being added to the respective aliquots. The optimal pH range was found to be broad, extending from 7.55 to 7.95 (40°C.) This was true for extracts from both males and females, the quantitative difference between the two remaining approximately constant at all pH. The peak, pH 7.8, falls within the range (pH 7.4-7.8) of values occurring in typical AG-peptidases (for example, Linderström-Lang and Sato, 1929; Duspiva, 1936; Holter and Doyle, 1938; Palmer

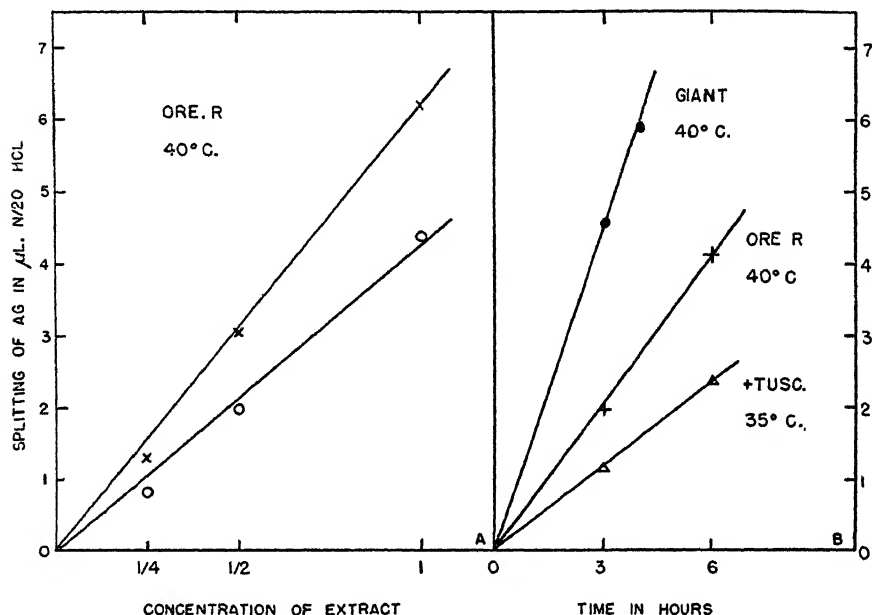


FIG. 3. Variation of AG-peptidase activity (A) with concentration of the enzyme extract, and (B) with reaction time. In A two extracts of salivary glands from Ore R 5 hour pupae (X), and larvae (O) were diluted to $\frac{1}{4}$ and $\frac{1}{2}$ concentration with glycerine buffer and the hydrolysis carried out at 40°C. for 4 hours. In B three extracts of standard concentration were used and the reaction carried out for varying times: ●, gt w^a Ore R 5 hour pupae; +, Ore R 5 hour pupae, and Δ, Tusc, late larvae.

and Levy, 1940). For most of the experiments a pH of 7.6 which falls well within the range of maximal activity was chosen in order to avoid spontaneous splitting of the substrate which even at 4°C. occurs perceptibly at higher pH after prolonged storage.

In the experiments in which concentrations of extract or reaction time was varied, the amount of substrate added was the same as that used in other experiments. At all times the substrate was present in excess. Over a fourfold range of extract dilutions, the relation between concentration and activity was linear (Fig. 3 A). If a complex of enzyme and some inhibitor present in these

extracts had been dissociated on dilution, a deviation from linearity would have been found at the higher concentrations. With the linear relation established, support is provided for the use of activity as a measure of enzyme content in extracts of comparable concentration. Within the experimental range, splitting was found to be proportional to reaction time in extracts from three different stocks (Fig. 3 B). Thus, with the concentration of substrate employed, the degree of hydrolysis is proportional to concentration of extract and time of reaction up to a titration value of $6 \mu\text{l. N}/20 \text{ HCl}$.

The usual temperature at which peptidase experiments are carried out is 40°C . The upper range for the normal development of *Drosophila melanogaster* occurs between 25 and 30°C . It was conceivable, although unlikely, that the enzymatic activity might increase at a lower temperature, one more physiological for *Drosophila*. In the time experiments just discussed, the Tusc extract was allowed to act at 35°C . It had previously been determined (*cf.* Table I, extracts 16 and 20) that Tusc and Ore R gave comparable values at 40°C . Consequently comparison of the curves from the two stocks, at the two temperatures (Fig. 3 B), shows an increase of activity at the higher temperature.

DISCUSSION

The attempt to analyze the enzymatic constitution of these crude extracts was necessarily based on the information available about the behavior of partially purified enzymes. Activators and/or inhibitors intrinsic to crude extracts may, however, distort the activity relationships which serve as a basis for identification of the enzymes. For this reason, substances which are present in the extracts and which might influence the enzyme activities deserve consideration.

Under the experimental conditions given, the substances most influential in determining the activity of a peptidase will be first the products of the catalyzed reaction, and second the substances (metals as coenzymes, inhibitors, etc.) affecting the enzyme directly either by way of influence on the active groups, or by non-specific reactions with the enzyme protein. The reaction products here—the free amino acids—are not present in the salivary glands or the extracts to any appreciable extent: the low titration value of the enzyme blanks agrees with the chromatographic analysis of LaCour and Drew (1947) on this point. For the metals, there is no analysis of the extracts available at present; and, of course, the absence of activation with certain of the metals may be due to their presence in sufficient quantities in the extract. The reducing systems have not been investigated in detail; there is no good evidence for the presence of much free —SH. Finally, as has been mentioned earlier, appreciable quantities of nucleic acid, about 0.05 mg. per ml. , appear to be present in the phosphate buffer extracts. The nucleic acids are known to inhibit the action of a different type of proteolytic enzyme, a carboxypeptidase

at pH 5 (Mims, Swendseid, and Bird, 1947). Hence, they merit attention when present in the enzyme extract. It may well be that the low values found for the endopeptidase characterized by cysteine-activated BAA splitting (pH 5.0) are due to the inhibitory effect of the nucleic acid in the extracts—a point that requires further investigation.

The activities exhibited by gland extracts toward the different exopeptidase substrates are evidence for the existence of a minimum of three enzymes: an AG-dipeptidase, a LG-dipeptidase, a leucineaminopeptidase; there may also be an aminopolypeptidase. It will be recalled that the rates at which the different substrates are split differ greatly. To facilitate comparison of the rates, the values of peptide hydrolysis by the different extracts can be averaged. If an arbitrary value of 1 is given to the rate for GGG, the approximate rates of the rest follow: GG, 2.5; LGG-Mn, 5; LG, 20; AG, 40. Clearly the high rate at which AG is hydrolyzed is distinctive and suggests that the enzyme is similar to the peptidase I of Linderstrøm-Lang (Linderstrøm-Lang and Sato, 1929; Linderstrøm-Lang, 1930). The other properties of the *Drosophila* enzyme support this conclusion: the rapid loss of activity in phosphate buffer without glycerine, the pH optimum at 7.8, and (Gailey and Johnson, 1941; Maschmann, 1941) the strong inhibition by cysteine. The data on activity of the enzyme when temperature, concentration of extract, or time are varied, are also consistent with the known behavior of AG-peptidases.

Since the hydrolysis of GG proceeded at a very low rate, it would appear possible that the AG-peptidase is responsible for this activity. A more probable alternative, however, is that a specific GG-peptidase (Smith, 1948 *b*) is involved. This cobalt enzyme is very labile and, if present, could have been largely inactivated during the prolonged extraction period. In the absence of experiments with Co^{++} activation, this possibility cannot be evaluated.

The data from the LG and LGG experiments require the assumption of two separate enzymes for their explanation. Were the typical leucineaminopeptidase (originally peptidase II of Linderstrøm-Lang, 1929; Smith and Bergmann, 1944; Smith, 1946; Berger and Johnson, 1939, 1940) responsible for both hydrolyses, the two substrates should have been split at the same rate, and activation by Mn^{++} should have occurred in both cases. However, with the *Drosophila* extracts, the rate of hydrolysis of LG-Mn is four times that of LGG-Mn. Moreover, LG hydrolysis is not activated by Mn while that of LGG is doubled. The conditions required by the assumption of a single leucineaminopeptidase are therefore not fulfilled. It might be assumed alternatively that the AG-dipeptidase is responsible for the LG splitting. But, aside from the objection to such an argument from the point of view of enzyme specificity, the different percentage inhibition with 0.01 M cysteine (90 per cent, AG; 60 per cent, LG) indicates the presence of two enzymes. A simpler interpretation would regard the LGG splitting as due to the usual leucineaminopep-

tidase and not to the non-Mn-activated lymphopeptidase (Fruton, Smith, and Driscoll, 1948); the LG splitting would then be attributed to a dipeptidase hydrolyzing LG, but not activated by Mn (Smith, 1948 *a*).

Finally, the presence of a still different enzyme may be indicated by the hydrolysis of GGG at a slower rate than LGG. A leucineaminopeptidase seems unlikely as the enzyme involved since the one studied by Smith (1948 *b*) does not act on GGG. This enzyme splitting GGG may possibly be similar to the aminopolypeptidase purified by Ågren (1945) and/or the lymphopeptidase of Fruton, Smith, and Driscoll (1948).

SUMMARY

1. Peptide-splitting enzymes have been studied in buffered glycerine extracts of larval salivary glands of three stocks of *Drosophila melanogaster*.

2. The ultraviolet absorption spectrum of the glycerine extracts indicates the presence of a considerable amount of nucleic acid.

3. Alanylglycine (AG), leucylglycine (LG), leucylglycylglycine (LGG), glycylglycine (GG), and diglycylglycine (GGG) are split by the gland extracts in descending order of activity.

4. Of the various metals added, manganese was the only one found to give clear cut activation and that only with LGG as substrate. Cysteine inhibited the splitting of both AG and LG.

5. Comparison of the data with those published indicates the presence in the extracts in descending order of activity (at pH 7.6, 40°C.) of at least four enzymes: an AG-dipeptidase, an LG-dipeptidase, a leucineaminopeptidase, and possibly an aminopolypeptidase.

6. Optimum conditions for the measurement of the enzyme splitting AG were determined. The pH activity and kinetic data are typical for an AG-dipeptidase.

7. An enzyme (probably cathepsin II) splitting benzoyl-L-arginineamide (pH 5.0) with cysteine activation was observed to occur with very low activity in gland extracts.

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PEPTIDASE INCREASE ACCOMPANYING GROWTH OF THE
LARVAL SALIVARY GLAND OF *DROSOPHILA*
*MELANOGASTER**†

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The study of enzyme synthesis during cellular growth is simplified by the use of a single type of cell whose growth proceeds under precisely defined conditions. For such experiments the larval salivary gland of *Drosophila melanogaster* is good material. The cells are of one kind, they increase in size without any division, and finally they break down during pupal metamorphosis. Hence the processes attendant on cell growth can be studied without the complications of mitosis, and the contrasting changes at cell breakdown can be followed. Moreover, *Drosophila melanogaster* is an organism in which the genetic and nutritional factors of growth can be varied with precision: the fly is a classic object of genetics, and it can be grown on a chemically defined medium (Schultz, St. Lawrence, and Newmeyer, 1946).

Following the exploratory work presented in the preceding paper (Patterson, Dackerman, and Schultz, 1949), the measurement of alanylglycine (AG)-peptidase activity could be used as an index of the content of AG-peptidase during growth of the salivary gland. Cellular growth, that is, the increase in size and/or number of constituent metabolic units, is largely a function of protein synthesis. The peptidases occupy a position of special interest in these processes. Not only may these enzymes be synthesized during the growth of the cell, but their function, even as hydrolytic agents, may be connected with the growth process.

This paper presents evidence for an increase in AG-peptidase content accompanying the growth of the gland, contrasted with a decline in measurable activity at the onset of histolysis. The increase in enzyme parallels the increase in total nitrogen content of the developing gland. A similar relationship between enzyme content and total nitrogen is evident in glands of different sizes, but at the same developmental stage. These results, when considered together with the data of earlier workers, particularly Linderstrøm-Lang and Holter and their group (see Discussion), lead to the conclusion that peptidase

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increase is a concomitant of cellular growth and suggest the possibility that these enzymes form an integral part of the synthetic system of the cell.

BIOLOGICAL MATERIAL

Characteristics of Salivary Glands

In spite of interest in the cytochemistry of the salivary glands aroused by the giant chromosomes of their nuclei, little is known of their physiology. Their development has, however, been studied both descriptively (Ross, 1939) and experimentally (Bodenstein, 1943). The larval salivary glands of *Drosophila* are paired structures joining into a common duct that opens into the pharynx of the larva. Full grown they are long and thin (Fig. 1), 1.0 to 1.7 mm. long by 0.16 to 0.25 mm. wide. Their average dry weight is about 5 $\mu\text{g.}$, their average volume 0.02 $\mu\text{l.}$ Each gland contains about 128 cells arranged about a central space, the duct. During larval life there is a thousandfold growth of the cells. With the approach of metamorphosis of the larva, the salivary glands begin to histolyze, finally breaking down completely except for the ring of imaginal cells destined to form the gland in the adult fly. These cells are adjacent to the duct and are easily removed when the ducts are cut off. The rest of the cells of the gland are all of one type. During development the cells at the tip are the first to attain a maximum size and degree of differentiation which is gradually approached by cells farther up the gland. The material used in the present work was limited to a late period of growth when the glands were large enough to permit easy removal of the adherent fat body. During this time most of the cells were in the final stages of differentiation.

The secretory function of the salivary glands of *Drosophila* is not well understood. Various staining techniques indicate an accumulation of secretory product at the end of the third instar. The secretion is trapped in the lumen of the gland at that time, possibly being given off in a mass during the subsequent formation of the puparium. After this time secretory globules are seen in the cells of the gland almost until definite histolysis sets in. Kodani (1948) has found that the secretion contains a protein material (10.5 per cent N) and has made a chromatographic determination of its amino acids.

Genetic Stocks

The material for these experiments is the same as that of the previous paper (Patterson, Dackerman and Schultz, 1949) but is described here more fully. Four stocks of *Drosophila melanogaster* were used. The first, a wild stock, + Tuscaloosa (Tusc), was only slightly inbred and was replaced in the experiments when on inbreeding it was discovered to contain a number of recessive mutants. The standard stock thereafter was a completely homozygous wild-type stock, + Oregon R (Ore R) (Bridges and Brehme, 1944); since its intensive use in this laboratory, it has been inbred for twenty generations by brother-sister matings. In addition to these two wild stocks, use was made of a sex-linked giant mutant (genetic symbol, *gt*; locus I, 0.01) in which about 20 per cent of the individuals have a prolonged larval life, pupating 2 to 3 days late as giant individuals (Bridges and Gabritschewsky, 1928). Two stocks of this giant mutant served as experimental material. The first (*gt Ore R*), made up to

contain Ore R autosomes, proved to be heterozygous for a second chromosome inversion (Cy 2R), and showed a variability which was too great for the later more precise experiments. The gt w^a Ore R strain was obtained from a series of crosses with stocks containing crossover eliminators marked by mutants. This resulted in the

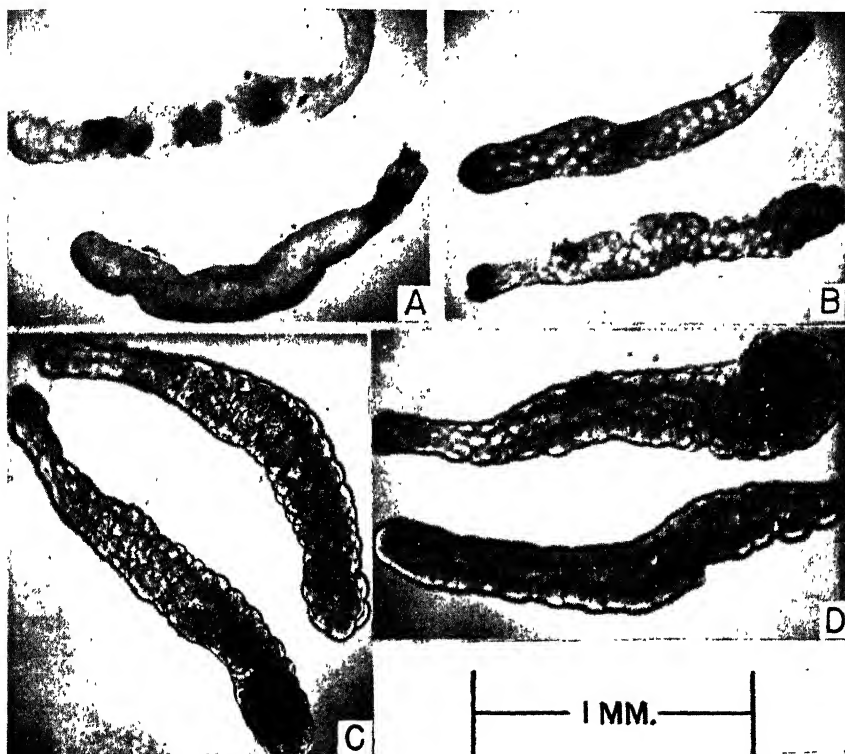


FIG. 1. Photomicrographs of larval salivary glands from prepupae of *Drosophila melanogaster*. A, pair of glands from an Ore R pupa 1 hour beyond stage ev. The AG-peptidase content of these glands is plotted in Fig. 2 at the points O₁ (gland at bottom) and O₂ (gland at top). Note the patches of dark cells in the upper gland indicating disintegration. B, C, D, pairs of glands from 5 hour prepupae of the gt w^a Ore R stock. Note the differences in size. B, from male non-giant prepupa, 120 hours after egg laying. C, from male giant, 185 hours after egg laying. D, from female giant, 168 hours after egg laying. $\times 38$.

production of a stock homozygous for the giant mutant and the sex-linked eye color apricot. Except for that small section of the X chromosome, the stock is isogenic with the inbred Ore R.

It is to be noted here that the difference between giant and non-giant flies in this stock is not genetic (the stock is homozygous, giants and non-giants breed alike) nor is it, as Bridges and Gabritschewsky supposed, nutritional. It must rather be con-

sidered as a variability in embryonic determination, leading to a delay in the time of release of the pupation hormone (Morgan, Bridges, and Schultz, 1936). The stock thus has the value of supplying individuals of identical genetic constitution, raised under identical environmental conditions, but still of different sizes at comparable physiological stages.

EXPERIMENTAL PROCEDURE

Culture of Larvae and Pupae

In a typical experiment, eggs were collected from a mass culture of male and female flies, all these the progeny of a single pair. Since it was necessary to be fairly precise in the timing of the larval and the pupal stages in certain of the experiments, eggs were collected from a 3 to 4 hour egg-laying period. In the case of the giants where special precautions were desirable, larvae were collected at hourly intervals as they hatched from the eggs which had been deposited on a yeasted molasses agar medium. The eggs or larvae were transferred to the normal medium used for raising the flies, the standard corn meal molasses agar mixture fortified with brewer's yeast and seeded with fresh yeast. Fresh yeast was added after 2 days and for the giants again on the 5th day (25°C.).

As the inbred stocks are now constituted and with standard culture conditions ensuring maximal feeding, developmental rates show little variability and indeed the results from generation to generation check quite closely. Formation of the prepupa occurred at about 120 hours (at 25°C.) after egg laying in both the Ore R and the *gt*^w Ore R non-giant larvae.

The stages of development (*cf.* Robertson, 1936) of the larvae and pupae from which the glands were dissected are defined as follows. "Late Larvae" (LL) are those third instar larvae that have crawled up the side of the culture bottle and are no longer feeding. Later prepupal stages are defined by the time elapsed at 25°C. from the moment (stage 0) at which the larva, with the pupal horns everted, no longer moves. Timing is necessary for the determination of the stages up to 3 hours after puparium formation. From 4 to 9 hours, identification is facilitated by a large gas bubble whose size and position in the prepupa is characteristic for each hourly stage. Later than 9 hours, one or another of the defined earlier stages was used for timing until pupation proper, with the eversion of the imaginal discs, occurred at about 12 hours. Glands from pupae in which the imaginal heads have just everted are at the "everted stage" (ev). The larval glands at this stage are at various degrees of disintegration, although still grossly whole in appearance (see Fig. 1 A).

Dissection

The dissection procedure was exactly as described in the preceding paper. All experiments were carried out in an air-conditioned, dust-proof room, and dissection was performed on a cold stage of a binocular microscope.

Measurements of Salivary Glands

1. *Volume*.—Since it was necessary to measure the volume of the glands, the procedure followed when single glands were used differed from that described for extracts

in that a pair of glands was photographed while in the drop of Ringer's solution. The whole operation of photography took about 1 minute, thus the glands did not warm up appreciably. The photographs served not only for volume calculations, but also as records of the condition of the glands.

An Argus camera carrying 35 mm. film was fitted to one ocular of the binocular eyepiece of a compound microscope (16 mm. objective, $2\times$ ocular), the other ocular being used for focusing. The area of the enlarged image of the negative was determined by using a planimeter and the greatest width was measured. The volumes were calculated assuming an ellipsoid shape for the glands ($V = 2/3 Ad$, where V is the volume, A the area, and d the diameter).

2. *Nitrogen*.—Nitrogen determinations in the range of $1\text{ }\mu\text{g.}$ are necessary for objects the size of the salivary gland. This amount of nitrogen may now be determined very accurately by the method of Brüel, Holter, Linderstrøm-Lang, and Rozits (1947) but this method was not available at the time these experiments were carried out. Our procedure was based on the Levy-Palmer (1940 *a*) decimicro adaptation of the Theorell (1928) sodium hypobromite method. It allows a direct determination of ammonia in the acid digest in the original tube and in this respect is simpler than the Carlsberg method which involves a transfer from tube to tube and a distillation of ammonia.

The decimicro method of Levy and Palmer was first used to determine total nitrogen in ten salivary glands. They were found to contain 2 to $8\text{ }\mu\text{g.}$ nitrogen. It was, therefore, necessary to refine the method and reduce the quantities by a factor of ten in order to determine nitrogen in one gland. Levy constriction type micro pipettes were used throughout and the final titration carried out with a type II Linderstrøm-Lang burette (Linderstrøm-Lang and Holter, 1940).

Details of the procedure used for the determination of total nitrogen in individual salivary glands are as follows: Each gland with the fat removed was transferred on a glass needle from Ringer's solution through a drop of double distilled water onto a small ($2\times 2\text{ mm.}$) coverslip. This was picked up with watchmaker's forceps and dropped into the digestion tube (Kimble glass precipitin tubes, 6 mm. diameter, 50 mm. long) containing $10\text{ }\mu\text{l.}$ water. Ten $\mu\text{l.}$ of $1:1\text{ H}_2\text{SO}_4$ was then added and the digestion carried out with the lower 5 mm. of the tubes resting in depressions in a heated and insulated aluminum block. The open ends of the tubes projected into a horizontal glass cylinder (1 inch diameter) through a slot ($\frac{1}{4}\times 12\text{ inches}$) cut into its lower side and thus were covered. Superoxol and potassium persulfate were used as additional oxidizing agents.

The total digestion time was about 8 hours at 210°C. Neutralization, addition of sodium hypobromite, acidification, and addition of solid KI were carried out in the original tube and at carefully timed intervals. The iodine released was immediately titrated with $N/20$ sodium thiosulfate. All operations were carried out in a scrupulously clean, air-conditioned, dust-proof room.

At the present stage of the work, nitrogen can be determined in the range 0.9 to $2.5\text{ }\mu\text{g.}$ with an error of $\pm 0.01\text{ }\mu\text{g.}$ Below $0.9\text{ }\mu\text{g.}$, while the routine error remains the same, the percentage error obviously increases. A few "outsiders" (see Brüel, Holter, Linderstrøm-Lang, and Rozits, 1947) also begin to appear. Nevertheless, the hypobromite method has been used in these experiments for determinations down to 0.4

μg. nitrogen. As seen from the figures given in Table I, the percentage variability in the total nitrogen values is about the same as that in the enzyme determinations and less than that in the volume determinations.

3. *Peptidase*.—Determination of the peptidase content of individual glands was carried out by the titrimetric micro methods of Linderstrøm-Lang and Holter (1940).

TABLE I
Peptidase Content, Total Nitrogen, and Volume of Salivary Glands in Various Stocks of Drosophila melanogaster

Stock	Ex- peri- ment No.	Sex	Stage	Age*	Peptidase†				Nitrogen				Volume				Peptidase Nitrogen
					M§	σ _M	Var.	N	M	σ _M	Var.	N	M	σ _M	Var.	N	
				hrs.	μl. HCl	%			μg.	%			μl. × 10 ³	%			
Tusc	1.08– 1.10	♀	LL	120	3.0 ± 0.3	31	11										
Ore R	2.01– 2.13		LL	120	3.2 ± 0.3	25	9						32 ± 3	19	5		
			4–5	120	5.3 ± 0.4	22	8		0.53				33 ± 3	21	7		9.9
			6–7	120	6.3 ± 0.5	22	7						40 ± 3	22	7		
gt w ^a	4.02	♀	5	120	4.0 ± 0.3	19	8		0.67 ± 0.03	13	9		32 ± 1	18	20		6.0 ± 0.5
Ore R	4.03		5	120	4.7 ± 0.2	15	8		0.67 ± 0.03	12	8		33 ± 2	24	20		7.1 ± 0.5
	4.03		5	167	9.2 ± 0.3	10	11		1.00 ± 0.03	9	12		60 ± 2	17	21		9.1 ± 0.4
	4.02		5	187	8.4 ± 0.2	5	5		1.11 ± 0.09	16	4		64 ± 2	13	17		7.5 ± 0.6
	4.03		5	187	8.7 ± 0.6	16	5		1.04 ± 0.02	5	5		58 ± 3	15	9		8.4 ± 0.6
Tusc	1.08– 1.10	♂	LL	120	2.5 ± 0.1	20	13										
Ore R	2.01– 2.13		LL	120	2.6 ± 0.3	30	5						30 ± 2	16	5		
			4–5	120	4.5 ± 0.4	19	5		0.47				25 ± 3	17	3		9.5
			6–7	120	4.6 ± 0.3	16	8						31 ± 2	15	8		
gt w ^a	4.02	♂	5	120	2.6 ± 0.2	16	7		0.54 ± 0.06	13	7		24 ± 1	15	20		4.9 ± 0.4
Ore R	4.03		5	120	3.0 ± 0.2	14	8		0.48 ± 0.03	16	9		26 ± 1	15	20		6.1 ± 0.4
	4.02		5	167	6.3 ± 0.3	10	4		0.81 ± 0.05	14	5		50 ± 3	18	8		7.8 ± 0.6
	4.03		5	167	6.2 ± 0.4	15	5		0.75 ± 0.03	12	7		47 ± 2	17	14		8.3 ± 0.7

* Hours after egg laying.

† Hydrolysis of 0.18 M alanylglycine by one gland in 4 hours at 40°C., pH 7.65, measured as the titration value; i.e., μl. N/20 HCl in 97 per cent alcohol. Titration values are read to the second decimal place; accuracy of blanks is ±0.08 μl. In Experiment 4.03 the time of hydrolysis was reduced to 3 hours and the readings corrected to the standard 4 hour period.

§ Mean (M); standard error of the mean (σ_M); coefficient of variability (Var.), σ_M/100; No. (N) of determinations.

|| From a single determination of total nitrogen in ten glands.

Details of the technique as applied to extracts of the glands were given in the preceding paper. The same procedure was carried out with the single glands except that instead of using an aliquot of gland extract as source of enzyme, here a gland was added to a 7 μl. aliquot of glycerine buffer (M/60 phosphate, 30 per cent glycerine) previously pipetted into the reaction vessel. In contrast to the experiments with extracts, the tissue was present during the enzymatic reaction.

In the experiments with the Ore R and Tusc stocks the glands were introduced into the buffer drop by means of a glass needle. Occasionally the glands stuck to the needle necessitating a return to the rinsing buffer drop to loosen them. This was found to cause a slight loss of enzyme. Therefore, in all the work with the gt w^a Ore R stock, the glands were transferred from the buffer drop to a small (about 2 × 2 mm. square) clean coverslip and the coverslip picked up with watchmaker's forceps and dropped into the tube (Holter and Doyle, 1938). Check experiments showed that this change in technique made no difference in the activities measured.

Immediately after a pair of glands was placed in the buffer drops, the two tubes were rapidly frozen (in dry ice-acetone) and thawed seven times. An additional tube containing a drop of buffer (reagent blank) was frozen and thawed with each alternate pair of glands. After an hour's extraction at room temperature the tubes were kept frozen over dry ice, until 5 minutes before substrate was added. (A time of extraction longer than 1 hour gave no increase in enzyme activity.) The tubes rested in holes in a disc of lucite cut to fit the small Dewar flask containing dry ice. No activity was lost after weeks of storage.

The process of freezing and thawing ensured sterility of the preparations, but did not produce cytolysis of the cells of the glands. In an attempt to ensure cytolysis, glands were placed in M/30 phosphate buffer, frozen and thawed, then extracted an hour before addition of 60 per cent glycerine (giving final concentration as above). Not only were the glands not cytolized, but the splitting of alanylglycine obtained was lower than that of similar glands extracted in glycerine buffer. Without the stabilizing effect of glycerine, the enzyme rapidly becomes less active, as is typical of this peptidase (Linderstrøm-Lang, 1929). Since standard results were obtained using glycerine buffer, the use of this procedure, which did not include cytolysis, seemed justified.

dl-Alanylglycine (0.18 M, pH 7.60 ± 0.05 at 40°C.) served as substrate for the peptidase determinations. In the previous work with extracts, the splitting of this peptidase was found to give a good index of peptidase content of the glands. Blanks were run as in the experiments with extracts except that with the single glands one gland of a pair was used for the "determination" and the other for the "enzyme blank." The enzyme blank values were found to be low and fairly constant, averaging 0.20 μl. N/20 HCl per gland (Ore R and Tusc) comparable to the values found in the gland extracts. In the final series of experiments, enzyme blanks were omitted, the second gland of a pair being used for a total nitrogen determination.

The reaction time in all experiments except those mentioned below was 4 hours at 40.0°C. Under the experimental conditions used (Patterson, Dackerman, and Schultz, 1949) degree of hydrolysis through a titration value of 6 μl. N/20 HCl was found to be proportional to concentration of extract and time of reaction. In the first experiment with the gt w^a Ore R stock, the splitting was found to exceed this value and the reaction time was subsequently cut to 3 hours.

Determinations of endopeptidase activity were carried out on single glands as described in the preceding paper for extracts. α -*N*-Benzoyl-*L*-arginineamide served as substrate and the reactions were carried out in citrate buffer at pH 5 with 0.01 M cysteine present. The reaction time was 20 hours at 40°C.

RESULTS

Three sets of experiments carried out under different biological conditions give information concerning the relation between peptidase and mass of the glands. The first set (measurements during the prepupal instar after food intake has ceased) deals with changes during the late stages of cell growth and differentiation and during the period of cell breakdown. The second set (measurements covering the late larval life of the giants) gives similar data for that period in which the cells are closer to the grand period of growth and in which the physiological state of the gland may still be conditioned by food intake. In the third group, genetically identical individuals of different sizes in the giant stock allow a comparison of glands at the same stage of development, but differing in mass. Considered individually, each set of experiments does not allow an unequivocal conclusion; collectively, however, they present a consistent picture of increase of peptidase during growth.

Peptidase Content and Total Nitrogen in the Larval Salivary Gland of the Oregon R Stock during the Prepupal Period

Fig. 2 presents measurements of the AG-peptidase content of salivary glands over a 14 hour period including the last stages of growth and the first stages of histolysis. Glands were taken from Ore R larvae just before puparium formation (stage L), and thereafter from prepupae at hourly intervals until eversion of the imaginal discs (pupation proper).

Curves of enzyme content in both males and females show a steady rise in the amount of hydrolysis of AG until the 6 to 7 hour period, the difference between the peak value and the initial measurement being statistically significant. Subsequently the peptidase content declines, with a considerable increase in variability. Both of these phenomena appear to result from the onset of histolysis, which is evident in the opaque patches on the glands after the 6 hour stage.

The correlation between histolysis and a lowered AG-peptidase content is illustrated by a comparison of the individual values given at points O_1 and O_2 , and X_1 and X_2 . The points O_1 and O_2 correspond to the peptidase contents of the two glands of a pair given in the photomicrograph in Fig. 1 A. The glands were dissected from a female pupa 1 hour after eversion. The gland at the bottom of Fig. 1 A shows little disintegration and it had a very high peptidase content, O_1 . Several opaque spots are clearly visible in the gland at the top and its peptidase content, O_2 , was significantly lower. In contrast, the greatest difference in peptidase content between glands of a pair in the stages up to 6 hours, *i.e.* before histolysis begins, was 5 per cent. The values X_1 and X_2 represent the highest and lowest peptidase contents found in glands from 11 hour male prepupae. X_1 is the value from a gland showing no visible sign of disintegration; X_2 , the value from a gland of similar size with

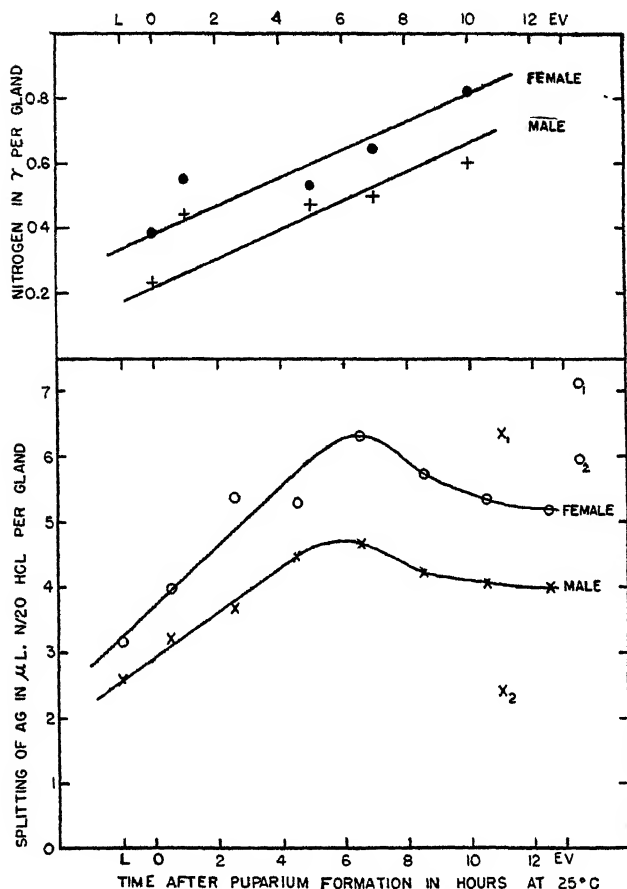


FIG. 2. AG-peptidase and nitrogen during the period of growth and breakdown of the larval salivary glands in the Ore R prepupa. Peptidase is plotted as the titration value; *i.e.*, the hydrolysis of AG in microliters of N/20 HCl in 4 hours at 40°C. The means of 5 to 12 determinations of the peptidase content of individual glands, grouped in intervals of 2 hours, are plotted in the lower graph. X_1 and X_2 represent the highest and the lowest of the individual male values at 11 hours; O_1 and O_2 , determinations made on two glands of a pair from an everted female pupa (see Fig. 1 A). Nitrogen determinations on ten glands are plotted in the upper figure.

many of the opaque patches that are characteristic of breakdown. In X_1 as in O_1 the enzyme content had increased over the values at earlier stages: disintegration of the "mature" cells had not yet begun, while the cells that were still growing continued to add to the enzyme content of the whole gland. In X_2 , where disintegration was well on its way, activity was decreased: the cells which had broken down subtract from the total possible enzyme content. It

seems likely, therefore, that per cell, the peptidase content increases until breakdown begins. Thus, the activity of the peptidase is not maintained at the time when the proteins of the salivary gland presumably become available for hydrolysis.

The fall in exopeptidase activity with disintegration of the gland is paralleled by a rise in endopeptidase activity (the ability to split benzoyl-*L*-arginineamide (BAA) at pH 5 with cysteine present). These experiments were carried out with the Tusc stock. When individual glands were used, from either late larvae or pupae in which the glands were still in the synthetic phase, no hydrolysis of BAA was found even with a 20 hour reaction time. But when glands from everted pupae were tested, that is glands that showed opaque spots indicating disintegration, a measurable but small hydrolysis of BAA occurred, averaging $0.30 \mu\text{l. N/20 HCl}$ per gland. This corresponds to the value found in extracts of glands (Patterson, Dackerman, and Schultz, 1949). Since the splitting with the single glands was low, two glands were used in many of the experiments and the time of extraction was cut to zero in order to keep the autolytic value (enzyme blank) small. Even so, this ranged from 0.06 to $0.84 \mu\text{l. N/20 HCl}$ when the hydrolytic value was 0.48 to $1.08 \mu\text{l. N/20 HCl}$. The variability in degree of hydrolysis corresponded to the variability in the degree of disintegration shown by the glands.

The interpretation of the changes with time in terms of actual growth requires the use of some index of total mass. Reduced weight, as used by Linderstrøm-Lang and Holter (1940), and the Cartesian diver balance of Zeuthen (1947) provide methods of weighing objects of this size but could not be used at the time of these experiments. Volume measurements provided an index of sorts, but with obvious weaknesses. Actually as may be seen in Table I (Experiments 2.01 to 2.13), the volumes found in this period were highly variable and showed no consistent trend. Since there are visible changes in the size of the lumen of the gland (secretion?), it was concluded that volume is not a satisfactory index of cell mass during the prepupal period. The measurement of total nitrogen appeared to be more useful in the case of the salivary gland in which no obvious large bodies of nitrogenous storage material are present. The nitrogen measurement has a major advantage from a theoretical point of view. Since the peptidases are probably largely protein, the total nitrogen gives a measure of all possible precursor nitrogen available for the synthesis of the enzyme.

Nitrogen values determined on groups of ten glands of the respective pupal ages are shown in the upper graph of Fig. 2. The points fall roughly on a line, with the exception of the 1 hour stage, at which a high value is found, possibly connected with a retention of the protein-containing secretion. During the prepupal period, the total nitrogen of the glands increases almost 100 per cent up to the time of breakdown. During the period of disintegration, it appears

that there is no appreciable release of nitrogen arising from the histolysis of protein and the diffusion of free amino acids from the gland. The enzyme blanks show very little change suggesting only a small increase in the concentration of free amino acids. Apparently the process of protein breakdown has not proceeded to completion at this stage.

It is evident that the peptidase and nitrogen content of the salivary glands in the prepupal period increase together, up to the onset of histolysis. After that time they diverge, the nitrogen continuing to increase while the peptidase declines. The interpretation offered is that the peptidase activity persists only in those cells in which histolysis has not yet occurred, whereas the nitrogen increase is due to continued growth in the "younger" cells of the gland.

*Peptidase, Volume, and Nitrogen Content of the Giant Salivary Gland
during Larval Growth*

The next step was to determine whether the increase of peptidase with total nitrogen is confined to the prepupal period, or is a general accompaniment of cellular growth of the gland. The grand period of growth occurs in the minute glands of the early larva which are difficult to deal with experimentally. But growth can be studied in the giant stock where the extra larval period provides individuals with large glands in which peptidase and nitrogen measurements can easily be made.

The survey of peptidase and nitrogen content made in the following experiments covers the latter third of the larval development of the giants produced in this mutant stock (gt Ore R). Larvae and/or pupae (stages 1, 2, 5, 8), in approximately equal number for each sex and stage of development, were collected at 10 and 20 hour intervals between 110 and 210 hours after egg laying. There are ordinarily two peaks of puparium formation in the larvae collected at one time from a given batch of eggs. One peak (non-giants) occurs at the normal time of puparium formation, about 120 hours (25°C.) after egg laying. The other peak (giants) occurs after a delay of 2 or 3 days. In the intervening time, the giant larvae can be collected. In this particular strain (see Genetic stocks) there were a few laggard non-giant pupae and there was a greater spread in the time of pupation of the giants than in the gt w⁸ Ore R stock used in subsequent experiments.

Mean values of peptidase contents of glands from three experiments are plotted in Fig. 3 A. Because of the variability in the stock, the data showed too great a scatter to permit a detailed study of the relations in the separate prepupal stages. These values were therefore grouped and used as a gross criterion of the final stage of larval development for a given time after egg laying. The data from the two sexes were also combined. It is clear that during the extra larval growth period of the giant, the peptidase content of the salivary gland rises with time. Thus a peptidase increase accompanying

growth is also characteristic of this larval period and is not merely a physiological peculiarity of the prepupal period.

When the individual values were inspected the data for the different prepupal stages allowed a rough comparison of the time relations of peptidase

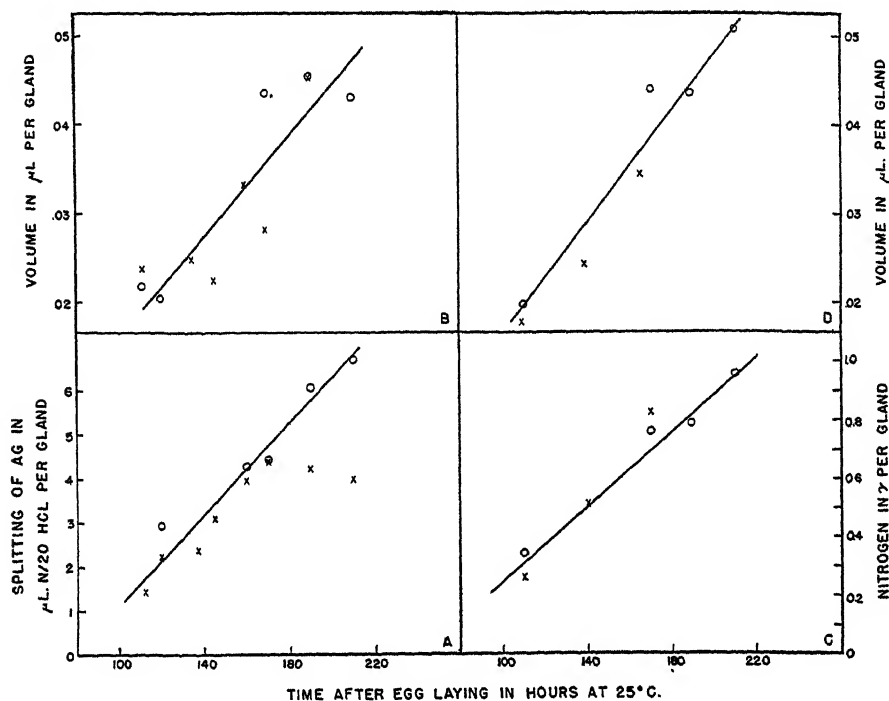


FIG. 3. Peptidase, nitrogen, and volume during larval growth of the salivary glands of the gt Ore R stock. Means of peptidase values (graph A) from larvae (X) and prepupae (O) are plotted separately but values from males and females and from the different prepupal stages are grouped. Each point represents the mean of 4 to 10 values included in ± 5 hours from the time after egg laying plotted. The mean values of the volumes from these same glands are plotted above in graph B. Graph C gives mean nitrogen values from a similar experiment. The volumes of the glands used in the nitrogen determinations are plotted in graph D.

change in the giant and non-giant individuals. Apparently, both in the giant and non-giant glands, the peptidase content is maximal at the 5 hour prepupal stage, thus agreeing with the results obtained on the Oregon R stock (Fig. 2). This result is of importance since the possibility existed that histolysis might begin earlier in the giants with their prolonged larval period.

After 180 hours, there are a few abnormally low larval values coming from individuals which eventually die without pupating. These glands may already

be undergoing some disintegrative changes of the type shown to occur in the normal gland just before the breakdown of metamorphosis. The low peptidase values would be consistent with such an onset of disintegration (see previous section).

The volumes of the glands from these experiments are given in Fig. 3 B. These measurements, made as described above, are approximate but sufficient to show that the peptidase increase is accompanied by an increase in volume. The volumes of the abnormal 190 hour larvae are high even though the peptidase is low, again suggesting that disintegration is lowering the peptidase value as in the pupal glands.

In order to correlate the peptidase and volume data with the total nitrogen of the glands, a similar experiment was carried out, in which nitrogen determinations were made on pairs of glands whose volumes were also measured. The results appear in Figs. 3 C and 3 D respectively. Both nitrogen and volume have increased as did the peptidase content and volume of the glands in the comparable experiment (Figs. 3 A and 3 B). The volume measurements are more variable than are those of total nitrogen; the latter are obviously a more accurate means of estimating the total protoplasmic mass. The volume measurements do show that variations in water content during this larval period must be on the whole minor to allow the general agreement with the nitrogen values.

It may be concluded, therefore, that during the larval growth of the gland in the giants, the increase in AG-peptidase is correlated with an increase in total nitrogen. But this increase, both in nitrogen and peptidase, occurs at a slower rate in the giant larva than in the Ore R prepupa previously examined. The time required for the peptidase content in the prepupa to double is only 6 hours whereas it is 50 hours in the larval giant gland. This difference poses a problem for future work. For the present, the important point is that in the two systems, where the absolute rates of increase are so different, the peptidase-nitrogen relations are similar.

Peptidase Content in Relation to Total Nitrogen in Glands from Giant and Non-Giant Prepupae

The measurements at the different stages of development described in the experiments of the two previous sections do not allow a discrimination between changes due to growth proper, and those due to differences in phase of the secretory cycle of the glands. In order to make such a discrimination, it seemed advisable to compare the peptidase-nitrogen relation in glands of different sizes but at the same physiological stage. Here differences in enzyme content would be directly related to cell size.

For this study the 5 hour prepupal stage of the giant and non-giant individuals was chosen. At this stage the peptidase content is at a maximum and the

fat body most easily dissected away from the gland. Males and females of the giants and non-giants provide a range of sizes sufficient to establish a relation between peptidase and nitrogen. As already pointed out, the existing data indicate that the change in peptidase content during the prepupal period is similar in these four types, allowing a valid comparison between them at the 5 hour stage.

The experiments (see Table I) were carried out on the rigorously inbred giant apricot Oregon R (gt w^a Ore R) stock. 5 hour non-giant prepupae were taken at 120 ± 2 hours after egg laying. Under optimum conditions the majority of the giant individuals in this stock have reached the 5 hour prepupal stage at 167 ± 4 hours. There are a few stragglers up to 187 hours. In Experiment 4.02 the female giants could not be collected at the proper time and the few later pupae were used. To allow comparison of individuals from the two different groups, 5 hour prepupae were collected at 167 ± 3 and 187 ± 3 hours (Experiment 4.03).

The experiments were further refined in that nitrogen determinations were made on one member of a pair of glands, peptidase determinations on the other. Volumes were calculated for all the glands used. Previous experiments had shown that the differences in peptidase content between members of a pair were not more than 5 per cent (Ore R), differences in total nitrogen values not more than 10 per cent (gt w^a Ore R), and differences in volume not more than 5 per cent (Ore R). The mean values obtained in two separate experiments of this kind (Experiments 4.02 and 4.03) are presented in Table I. The individual values for Experiment 4.03 are plotted in Fig. 4, the ordinates being the amount of hydrolysis of alanylglycine found in one member of a pair of glands, the abscissae being the total nitrogen found in the other member of the pair.

It is evident that in both experiments, the higher the total nitrogen, the higher the peptidase content. However, the ratio between the two quantities is not constant: at the higher nitrogen values of the giant groups, the average peptidase-nitrogen ratio is over 8, as compared with the lower ratio around 7, of the non-giant groups. This difference in ratio may indicate a situation in which part of the nitrogen increases directly with the AG-peptidase, while the remainder is unrelated to this enzyme. Further data defining more closely the nature of the curve of enzyme increase during development are clearly needed before such a discussion could be profitable. The emphasis at present is placed upon the fact that in glands of different sizes, at the same physiological stage, the AG-peptidase content of the gland goes along with the increase in mass as measured by total nitrogen.

Thus from the three different sets of experiments, the same conclusion follows: the higher the total nitrogen, the more peptidase, until histolysis begins. It seems a reasonable conclusion from the experiments of the present section that

this increase is concomitant with the "true" growth of the gland and is not merely a reflection of changes in the secretory cycle. It is difficult to conceive that the parallelism between peptidase content and total nitrogen would otherwise be maintained under the three sets of conditions. Only if secretion were continuous and formed a constant proportion of the total nitrogen could such a result be obtained, and in that case the secretion would be essentially a con-

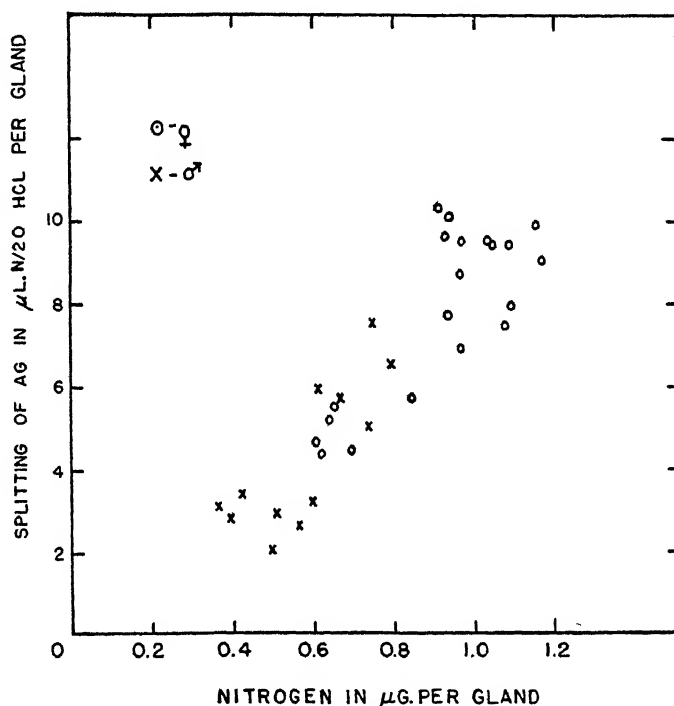


FIG. 4. The relation between peptidase and nitrogen in 5 hour prepupal glands from gt w^a Ore R males (X) and females (O). Peptidase determinations were carried out on one gland of a pair, nitrogen determinations on the other.

stant component of the cell system. Actually what evidence there is, as already stated, points to a peak of secretion just before puparium formation. Yet the above data give no indication of any major effect of this process on either the peptidase or the nitrogen.

Relation between Peptidase Content and Genetic Factors

It is obvious that enzymes with the high degree of specificity shown by the peptidases are suitable material by means of which a study of the relation between genes and enzymes can be approached. The present study, while tangential to this objective, gives some information of a preliminary character.

Knowledge of the enzyme spectrum of the salivary gland should make it possible to screen the different mutants in *Drosophila* for differences in content of specific enzymes. Since this paper is concerned with only one enzyme, and mainly with only four genotypes (males and females of the giant and Ore R stocks) these data could only give evidence of striking quantitative differences.

The information is most complete concerning these relations between male and female. The peptidase content of salivary glands of males is consistently lower than that of the females in all the stocks studied. The data of Fig. 4, already discussed, show that this low value is correlated with a correspondingly low nitrogen content. Thus it appears that the difference in peptidase content between male and female is the result of a difference in mass, corresponding to the well known fact (Dobzhansky 1929) that the cell size of the male is smaller than that of the female.

The interdependence of peptidase content, total nitrogen, and volume measurements as between males and females is strikingly borne out by a comparison of the sets of values from the different experiments. When the values for males are plotted against the corresponding ones for females (Fig. 5) all these points for peptidase, volume, and nitrogen fall on the same straight line: the ratio (1:1.35) between males and females is constant for all points. The sexual difference in the peptidase content has the same ratio as the difference in mass.

The graph discussed contains one additional set of points which necessitates a slight digression. The values obtained in experiments using extracts of the Ore R and Tusc stocks (Patterson, Dackerman, and Schultz, 1949) fit on the same part of the line, *i.e.* have the same absolute values, as those obtained from experiments in which the whole gland was present, showing the completeness of extraction of the enzyme.

The dependence of male-female relationships on differences in mass reinforces the main thesis of this paper. It does not give indications of genetic influences in a specific way on peptidase content. A difference deserving some note was found between two strains of the first stock of the giant mutant used (gt Ore R), in which only the autosomes were of Ore R ancestry. One of these strains gave high peptidase values, the other lower values and showed a much higher variability. It was found that the variable stock actually was heterozygous for an inverted section of chromosome II, introduced into the stock by an undetected rare crossover during its preparation. The use of the stock was thereupon discontinued; but the difference due to change of chromosome composition is pertinent.

A more definite difference, which holds promise for future work, is found in the comparison of non-giants from the gt w^a Ore R stock with individuals from the Ore R stock having a similar nitrogen content. The Ore R peptidase values are high relative to the non-giants although they agree fairly well with earlier

values on another wild type stock, Tusc. The AG-peptidase-nitrogen ratio is, however, the significant quantity (see Table I), being lower in both males and females of the non-giants (120 hours) of Experiments 4.02 and 4.03 than it is the Ore R individuals of a similar age and nitrogen content in Experiments 2.01 to 2.13. The meaning of these differences is a problem to be analyzed in

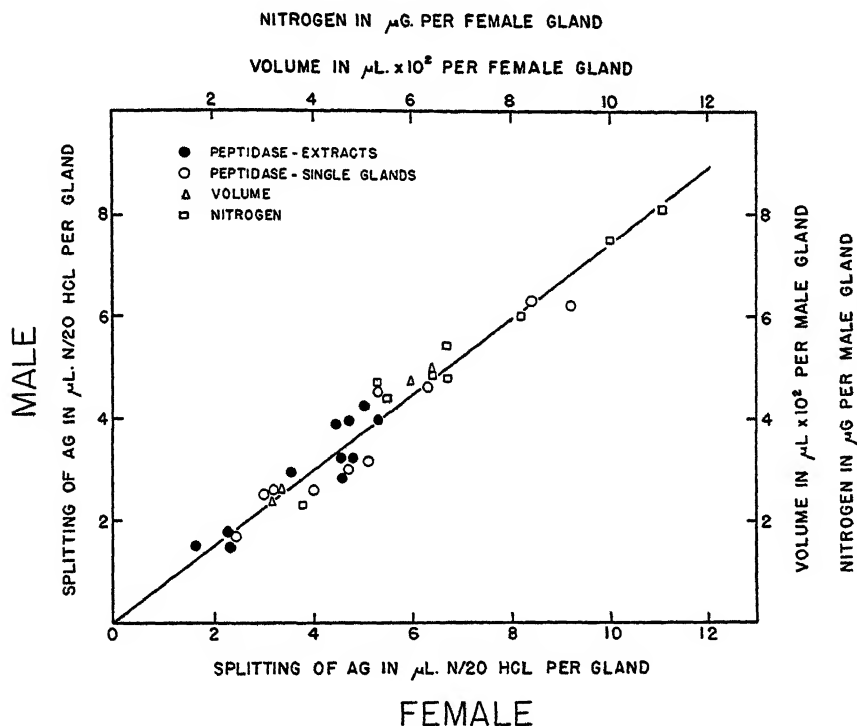


FIG. 5. Male:female relationship in peptidase, nitrogen, and volume determinations made on salivary glands. The line represents $\bar{y} = 1.35 \bar{x}$. The points plotted are means of determinations made on glands of various stages from the Tusc, Ore R, and gt w^a Ore R stocks. The peptidase values for extracts of glands from the Tusc and Ore R stocks were taken from data of Patterson, Dackerman, and Schultz (1949).

detail, on separate isogenic gt and w^a stocks, upon which measurements of the course of peptidase increase must be carried out. Thus, in addition to the general relation to growth, the closer study of this material should hold interest for chemical genetics.

DISCUSSION

Histochemical estimations of peptidase content have the limitations noted by their originators (see Linderstrøm-Lang and Holter, 1934). An assay of

the total amount of enzyme content is obtained by measuring its activity as evidenced in the hydrolysis of a specific substrate under defined conditions. Such determinations do not allow conclusions to be drawn about the activity of the enzyme in the cell. Yet barring intracellular changes in the ratio of enzyme to inhibitor or activator, measurements showing increase in enzyme activity are *prima facie* evidence of enzyme synthesis.

The present data, showing peptidase synthesis in cells whose growth is uncomplicated by mitosis, complement the earlier work on other organisms. In the histochemical analysis of the barley root (Linderstrøm-Lang and Holter, 1932; Bottelier, Holter, and Linderstrøm-Lang, 1943) an attempt was made to distinguish between the possible rôles of the peptidases in cell division and in growth. In the root tip, maximum enzyme activity per unit mass appeared in the zone, not of maximum mitotic rate but in the region just beyond, where total nitrogen per unit mass is maximal and where presumably increase in "active protoplasm" is greatest. It was this correlation which led Linderstrøm-Lang and Holter (1932) to suggest that peptidase content is high in regions of active intracellular synthesis. This thesis received support from the later work of Levy and Palmer (1940 *b*) who showed a parallelism between increase in total nitrogen and AG-peptidase in the chick, as well as from that of Pickford (1943), who correlated AG-peptidase content with the extractable protein in the salamander embryo. Similarly, Dumm (1943) found a peak of peptidase content corresponding to the period of rapid growth of the fetal liver in the rat. In all these cases, the intermixture of dividing and non-dividing cells and the presence of cells at different stages of division blurred the picture.

Attempts to achieve greater precision, carried out by Linderstrøm-Lang, Holter, and their group, took two directions. One was the analysis of the cytological structures to which the AG-peptidase might be bound. The peptidase activity of the cells studied was not bound to any of the larger granules but seemed distributed uniformly throughout the "active protoplasm" (Holter, 1936; Holter and Kopac, 1937). Thus the attempt to study peptidase activity by localizing its function gave a result which, while consistent with the idea that peptidases have to do with synthesis, did not help to define their rôle.

The second approach was a classic series of experiments designed to analyze the changes in enzyme distribution during early cleavage in marine eggs, where possible relations to the processes of differentiation could be studied (Doyle, 1938; Holter, Lanz, and Linderstrøm-Lang, 1938; Holter and Lindahl, 1941). Again the AG-peptidase was distributed in proportion to the "active protoplasm" and again no association with any specific function or structure appeared. Of major importance for the present discussion, however, is the finding that no increase in peptidase content occurred during the early cleavages. This period of development is precisely the time during which no increase in

nitrogen occurs (Ephrussi and Rapkine, 1928) and is an example of a biological system in which reproduction of the chromosomes takes place at the expense of components already present in the egg; *i.e.*, no new cytoplasm is formed. Thus, in the sea urchin egg, the formation of chromosomes need not be accompanied by increase in peptidase content.

The sea urchin data are reinforced by the findings of Doyle and Patterson (1942) on the protozoan *Didinium*. Two divisions after feeding, the total content of AG-peptidase in the daughter cells remained equal to that of the original parent. Thus, in these cases, division is accomplished without increase of either total mass or peptidase content. Conversely, the present studies of the salivary gland show that an increase of cell size without division is accompanied by an increase in the enzyme content. A consistent pattern appears: the association of AG-peptidase with the processes of synthesis in the cell.

The work of Duspiva (1942) on AG-peptidase during the growth of the frog oocyte provides an important complement to the argument. Here is still another type of cell, one in which cytoplasm is being formed to support the subsequent rapid embryonic cleavages. In this material the various phases of growth are seasonal and can be separated. Measuring the peptidase content per unit cytoplasm, Duspiva found a rise in concentration at the onset of yolk synthesis, then a maximum, with a later decrease explained by "dilution" of the cytoplasm by inert yolk. A recalculation of his data can be made in terms of the total peptidase per cell. On this basis, the increase in peptidase content continues to the end of the growth period as does the synthesis of yolk. In this case, as in the case of the salivary gland, the increase in enzyme is correlated with other syntheses in the cell.

The concept of an "active protoplasm," of which the peptidase content is characteristic, was used by Linderstrøm-Lang and Holter to interpret their data. On closer examination, this implies that the enzyme increases together with the functional working of the cell. Perhaps the simplest hypothesis is that the peptidase is part of a system of coupled reactions forming a unit of protein synthesis in the cell.

This suggestion, that AG-peptidase forms part of a biological unit of synthesis, requires examination in terms of other components of such a hypothetical system. The nucleoproteins deserve first consideration in view of the suggestions that they have to do with protein synthesis (see Caspersson, 1947; Brachet, 1947). A series of analyses of ultraviolet absorption spectra of cells of the salivary gland at different stages in the larval period (Schultz and Caspersson, 1938) is relevant to this question. The changes in the spectra could be interpreted as being due to a decrease in the pentosenucleic acid (PNA) to protein ratio of the salivary gland cytoplasm as the cell approached maturity. The differentiation of the cell from its embryonic state entails a decrease in

the nucleic acid concentration, even though the total amount increases. It is not possible as yet to correlate the peptidase data with the nucleic acid information, since different stages of the life cycle of the gland are covered by the two sets of data. It is essential to establish a precise correlation of the peptidase with the nucleoprotein data, of the sort carried out by Thorell (1947) for nucleic acid and hemoglobin synthesis in the blood cell.

In the plant root there is some evidence of a correlation between high PNA in the cytoplasm and high peptidase content. The data of Bottelier, Holter, and Linderstrøm-Lang (1943) show, for the barley root, a region of maximum peptidase content in the young cells, and a gradual fall in the older cells of the root. It is well known that the basophilic character of the cytoplasm decreases in the older cells, thus paralleling the decrease in peptidase content. For the onion root, the ultraviolet absorption data (Caspersson and Schultz, 1939) show a change from a high cytoplasmic nucleic acid concentration in the young cells to a very low concentration in those cells that have reached their complete differentiation.

Additional correlations between growth, peptidase content, and cytoplasmic PNA content can be found in other materials. In the sea urchin, according to Schmidt *et al.* (1948), the PNA content is constant during early development—the same period as that in which the peptidase is constant according to the Carlsberg group. Conversely, in such cases as the chick embryo and the rat liver, where the AG-peptidase content increases (see above) the PNA content increases (Caspersson and Thorell, 1941; Davidson, 1947; Novikoff and Potter, 1948). Such correlations may, of course, mean nothing more than the platitude that the growth of a cell involves the increase of its parts. Consideration of the behavior of other systems should provide a means of distinguishing real from spurious associations in growth processes. The concept, therefore, of a unit of synthesis involving the nucleic acids and a group of enzymes, including the peptidases, may prove useful in orienting further experiments particularly in connection with the stimulating hypothesis of Brachet and Chantrenne (1944) that the granules of the cytoplasm themselves may have a developmental history. On such a basis the apparent uniform distribution in cytoplasm of AG-peptidase and other peptidases may only mean an association with the smallest of the granules, those which in the Brachet and Chantrenne hypothesis are considered to be the progenitors of the larger and more elaborate ones.

The contrasting behavior of the AG-peptidase and the endopeptidase studied deserves some comment. The appearance of activity towards BAA in the histolyzing salivary gland suggests that the endopeptidase may function in the process of cell breakdown. The finding of Zamecnik and Stephenson (1947) can be interpreted in this light. They found appreciably higher activity towards BAA in hepatoma than in the control adult and fetal liver although the dipep-

tidase activities were comparable in the three tissues. In the tumors there is not only the possibility that necrotic tissue may have been included, but there is also present the type B cell of Caspersson and Santesson (1942), which is at a stage along the road to breakdown. It is possible that the endopeptidases begin to function at a time when protein denaturation has exposed susceptible peptide linkages.

The preceding discussion, biological in nature, has left untouched the question of the actual mechanism by which the peptidases may be related to protein synthesis. The recent discussions of this problem, those of Fruton excepted, have tended to minimize the possibility that the synthesis of peptide linkages is mediated by peptidases. The preferred view looks to phosphorylations to provide the requisite energy. It should be pointed out, however, that the peptidases may play a rôle of importance simply by carrying out their hydrolytic functions. The AG-peptidases (and also the other exopeptidases) may have the function of permitting amino acid transport from protein to protein, as members of a series of coupled reactions, thus playing a part in the exchanges shown to occur by the work with isotopes (Schoenheimer and Rittenberg, 1940). In this way they would permit the mobilization of specific amino acids at the particular places in the cell where the presence of energy-carrying phosphate groups allows synthesis to proceed.

SUMMARY

1. The larval salivary gland of *Drosophila melanogaster* offers an opportunity to study growth in a tissue in which no cell division occurs but in which the cells increase in size.

2. Measurements of alanyl-glycine (AG)-peptidase content have been made in three stocks of *Drosophila melanogaster* at different growth stages of the larval salivary gland, and have been correlated with its total nitrogen and volume.

3. During the prepupal instar, the AG-peptidase content of the gland increases parallel with total nitrogen but decreases when histolysis of the gland begins. Conversely, a benzoyl-L-arginineamide-hydrolyzing endopeptidase is not measurable until histolysis sets in.

4. In the final larval growth period of a giant mutant, there is a concomitant increase in peptidase, total nitrogen, and volume of the gland.

5. A similar association of peptidase content and total nitrogen is found in comparing glands of different sizes from the giant stock, at the time of maximal peptidase content in the prepupa.

6. The data are interpreted as evidence for an association of AG-peptidase with growth of the cells in the gland. This agrees with the earlier interpretation by Linderström-Lang and Holter of data obtained from study of more complex tissues.

7. A survey of the available measurements of peptidase content in other

organisms shows that wherever an increase of cell substance occurs, peptidase content increases. Conversely, peptidase remains constant where cell division is unaccompanied by an increase of cell substances.

8. The joint association of peptidases and pentosenucleic acids with protein synthesis is pointed out.

9. The possibility is considered that peptidases may be essential parts of a unit in which coupled reactions necessary for protein synthesis occur. The rôle of the peptidases in this system is discussed. They may act either synthetically to form new peptide linkages (problematic), or hydrolytically to mobilize the necessary specific amino acids.

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MUTANTS PRODUCED BY X-IRRADIATION OF SPORES OF *CHAETOMIUM GLOBOSUM* AND A COMPARISON WITH THOSE PRODUCED BY ULTRAVIOLET IRRADIATION*

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INTRODUCTION

Mutations have been produced by irradiating spores of *Chaetomium globosum* with soft x-rays from a Shearer tube operating on 12 to 15 kv. and with harder x-rays from a dental tube operating on 62 kv. In this paper the mutations produced by x-rays are classified and a general comparison made with those produced by ultraviolet irradiation (previously described in papers by McAulay, Plomley, and Ford, 1945; Ford, 1946; Ford, 1947; McAulay and Ford, 1947; and Ford, 1948).

In the later papers on ultraviolet experiments the mutants have been classified into two broad groups. The same division is made in this paper.

(a) *Lethal Mutants*.—These are mutants in which the growth of the colony ceases before it has become visible (Ford, 1948). Upon microscopic examination these colonies are found to possess marked characteristics easily recognizable. They are produced by ultraviolet irradiation, visible light, and x-rays; they also occur in the progeny of certain macroscopic mutants. They are produced in up to 100 per cent in some irradiation experiments. Less than 1 per cent occur in the controls.

(b) *Macroscopic Mutants*.—These are visible mutants in which the growth rate and form vary from the normal colony. Up to 62 per cent occur with short ultraviolet irradiation; in x-ray experiments 15 per cent occur; and with long ultraviolet and visible wave lengths up to 19 per cent are produced. With these also, less than 1 per cent occur in controls.

Detailed experiments on the relation between dose in roentgens and the percentage lethal mutants have been made with x-rays from the dental tube. It has been found that the production of the mutants is consistent with five quantum hits. It would appear from the form of the curve discussed later that in x-ray experiments more quantum hits are required than the two quantum hits consistent with the curves obtained for ultraviolet mutant production (McAulay, Ford, and Dobie). A difference in position of the sensitive

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spot which absorbs the radiation in different spores may result in too low a figure for the number of quantum hits (2) obtained with mutant production in ultraviolet experiments.

In the case of the macroscopic mutants produced by x-rays, the most striking feature is the scarcity of the mutant *K* which is produced selectively and in large numbers by short ultraviolet wave lengths (McAulay, Plomley, and Ford, 1945; Ford, 1946). In this respect the results obtained with x-rays are similar to those obtained with long ultraviolet wave lengths, which is a striking result suggesting that the production of the *K* is restricted to a limited region of the electromagnetic spectrum. An analysis of the pigmented mutants suggests that more are produced by x-ray irradiation of the fungal spores of *Chaetomium globosum* than are produced by ultraviolet irradiation.

EXPERIMENTAL

Spores of the fungus *Chaetomium globosum*, Fld, were used. As stated in earlier papers from this laboratory (1945, 1946, 1947), Fld is a stable mutant which was produced in an early ultraviolet irradiation experiment. In these papers the ultraviolet apparatus and experimental procedure were described.

In the x-ray experiments the spores were spread with a brush on a circular area of celluloid half an inch in diameter. Approximately 4,000 spores were spread for both experimental and control samples. After irradiation the colonies were obtained by single spore and dilution plate techniques which have been described in detail by McAulay, Plomley, and Ford (1945). Lethal mutants were all of single spore origin. Single spores were examined daily for germination, until it was clear that either the colony became visible or could be classified with certainty as a lethal mutant. In some cases the examination was continued up to 5 or 6 days. Visible colonies were plated on 2 per cent malt agar, each colony in a separate Petri dish, and incubated at 28°C. After about 3 weeks, an analysis was made of the matured colonies for macroscopic mutants.

In experiments on the dose-per cent mutant relation, spore samples were taken from the irradiated and control spreads, 100 spores being plated singly from each irradiated sample and 50 from each control sample. In obtaining macroscopic mutants, both the single spore and dilution plate techniques were used. The lethal mutants were counted and expressed as a percentage of the numbers of germinated spores; the macroscopic mutants were expressed as a percentage of the number of visible colonies.

X-rays were obtained from a Watson Victor S.F.1 dental x-ray unit. Special precautions were taken to accurately locate the x-rays at the distance of 0.45 inch from the focal spot. Calibration figures, supplied by Dr. Eddy of the Commonwealth X-ray and Radium Laboratory, for this x-ray unit, show that the air dose at a distance of 0.45 of an inch from the focal spot is 28,200 roentgen units per minute. Eddy's figure checks fairly well with measurements made with a Victoreen metre borrowed from the Royal Hobart Hospital. It is calculated that the dose required to produce 50 per cent lethal mutants is 94×10^4 r. Accuracy is not claimed for the measurements of dose shown in this paper which are given for relative value only. Lea (1946) states that the ionization in tissue is 1000 times that in air.

Soft x-rays were obtained from a Shearer tube unit, which was constructed in this laboratory. Unfortunately no measure of dose was made. The macroscopic mutants which were produced by soft x-ray irradiation were similar to those produced by x-rays from the dental unit.

The Relation between Dose and Numbers of Lethal Mutants with X-Rays

Table I shows the relation between dose and numbers of lethal mutants. The first column gives the dose in roentgens, column 2 gives the numbers of single germinated spores, column 3 gives the ratio of the percentage experi-

TABLE I
Showing Relation between Dose and Numbers of Lethal Mutants with Hard X-Rays

Dose	No. of single germinated spores	Ratio $\frac{\text{Experimental germination}}{\text{Control germination}}$	No. lethal mutants	Lethal mutants
<i>roentgens</i>				<i>per cent</i>
9.4×10^3	329	1.02	4	1.21
18.8×10^3	373	0.99	3	0.81
37.6×10^3	378	0.98	26	6.87
47.0×10^3	77	1.6	3	3.89
56.0×10^3	302	1.05	45	14.9
75.2×10^3	446	0.87	96	21.5
94.0×10^3	96	0.95	48	50.0
112.8×10^3	62	1.04	38	61.2
131.0×10^3	64	1.2	58	90.6
164.5×10^3	61	0.96	56	91.63
188.0×10^3	55	1.67	47	85.45
235.0×10^3	35	1.39	33	94.28
329.0×10^3	10	0.57	10	100
Total experi- mental.....	2,189	—	467	21.7
Total controls....	1,353	—	1	0.07

mental germination to control germination, column 4 shows the number of lethal mutants, and column 5 the percentage lethal mutants. A total of 467 mutants was obtained from 2,189 germinated spores. Experiments using a wide range of dose were made extending from 9.4×10^3 roentgens, which gave 1.2 per cent lethal mutants, to 329×10^3 roentgens which gave 100 per cent lethal mutants. It will be seen that only one (0.07 per cent) lethal mutant was obtained in 1,353 control colonies. Column 3, giving the ratio of experimental to control germination shows that for almost the whole range of dose from 9.4×10^3 to 235×10^3 roentgens the ratio is approximately constant. The ratio of experimental to control germination is low (0.57 per cent) only at the highest dose used.

Fig. 1 shows the percentage lethal mutants plotted against dose in roentgens. The percentages of lethal mutants are shown as circles on the graph and the standard deviation of each circle or point is given as a vertical line. Theoretical curves for one, two, five, and eight quantum hits are shown on this figure. These curves are taken from Madam Curie's paper (1929). The percentages obtained in the dose-mutant relation experiments made at the ultraviolet wave length 2804 are shown for comparison as crosses in this graph. It will be seen that the x-ray points fit most closely to the five quantum hit curve.

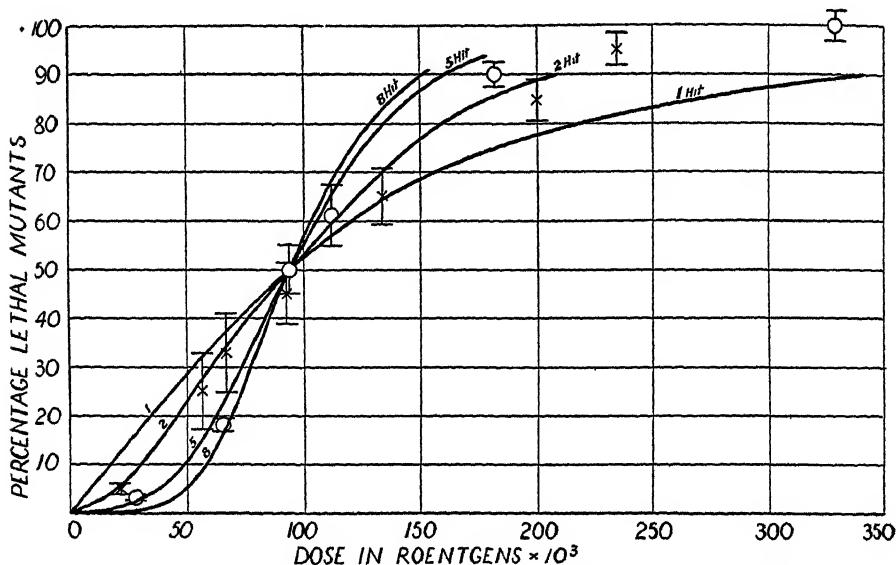


FIG. 1. Showing experimental points for percentage lethal mutants obtained with x-rays from the dental unit plotted against dose in roentgens as circles, and points for the ultraviolet wave length 2804 shown for comparison as crosses. Theoretical curves for one, two, five, and eight quantum hits are plotted.

The points on the most important part of the curve, that is at its beginning, have a very low standard deviation, and the fit to the five quantum hit curve is extremely close. This suggests that five quantum hits are required on the sensitive spot of the spore in order to produce a lethal mutant. The points for the 2804 ultraviolet wave length, which are taken from an unpublished paper by McAulay, Ford, and Dobie, are consistent with a two quantum hit mechanism.

The form of the curves in Fig. 1 suggests that x-rays require more quantum hits to produce a mutant than ultraviolet irradiation. However, as mentioned in the paper by McAulay, Ford, and Dobie, the two quantum hits thought to be consistent with ultraviolet mutant production may be too low owing to the varying depth of the sensitive spot in the spores.

Fig. 1 shows that the one and two quantum hits are quite inconsistent with mutant production by x-rays. Table II gives results plotted in Fig. 1, the standard deviation $(pq/n)^{1/2}$ being shown in column 4 of the table.

Production of Macroscopic Mutants by X-Rays and a Comparison with Those Produced by Ultraviolet

Table III shows results of experiments carried out with soft x-rays, hard x-rays, short ultraviolet wave lengths, and long ultraviolet and visible wave lengths. The average percentage of lethal mutants in experiments shown in the table is between 60 and 74 per cent, the actual figures being listed in row 12 of the table.

TABLE II

Showing Standard Deviations of Six Average Percentages of Lethal Mutants Obtained with X-Rays from a Dental Unit

p	q	n	$(pq/n)^{1/2}$	Average dose
Average of lethal mutants	$(100 - p)$	No. of germinated spores	Standard deviation	
<i>per cent</i>				<i>roentgens</i>
3.2	96.8	1157	0.517	28.2×10^3
18.2	71.8	748	1.32	65.6×10^3
50.0	50.0	96	5.1	94.0×10^3
61.2	38.8	62	6.23	112.0×10^3
90.2	9.8	215	2.42	182.0×10^3
100	0	10	3.16	329.0×10^3

The most striking feature of the comparison of the macroscopic mutants produced by x-rays with those produced by ultraviolet wave lengths is the almost entire absence of the *K* mutant in x-ray experiments. Large numbers of the *K* mutant (see row 6) are produced by short ultraviolet wave lengths, (also papers by McAulay, Plomley, and Ford, 1945; Ford, 1947; and McAulay and Ford, 1947), but few are produced by other irradiations. At 3132 the shortest wave length included in the long ultraviolet and visible wave lengths, 0.76 per cent *K* mutants are produced. If these are omitted from the long ultraviolet and visible wave lengths (column 4), only 0.64 per cent *K* mutants remain in this group. Only two *K* mutants were produced in all x-ray experiments, one in soft x-rays, and one in hard x-rays, giving a percentage 0.12 *K*'s produced by x-rays. Compared with these low numbers, over 400 (31 per cent) *K* mutants were obtained at short ultraviolet wave lengths. This evidence suggests that the production of the *K* is confined to a limited region of the electromagnetic spectrum.

When the mutants "other than *K*" produced by x-rays, are compared with those produced by ultraviolet and visible irradiation, it is seen that the percent-

ages of mutants in all four groups are similar. They are 7.6 per cent for hard x-rays, 9.9 per cent for soft x-rays, 6.6 per cent for short ultraviolet wave lengths, and 7.2 per cent for long ultraviolet and visible wave lengths. Many of the mutant types produced by x-rays are identical with those produced by ultraviolet irradiation.

TABLE III

Showing the Macroscopic Mutants Produced by X-Rays and a Comparison Made with Those Produced by Ultraviolet and Visible Irradiation

	Hard x-rays from a dental unit		Soft x-rays from a Shearer tube		Short ultra- violet wave- lengths from 2482-3025 Å.u.		Long ultraviolet and visible wave- lengths from 3132-4047 Å.u.	
	No.	per cent	No.	per cent	No.	per cent	No.	per cent
1. No. of experiments.....	5		6		13		12	
2. Total experimental colonies.....	588		686		1330		1409	
3. Total control colonies.....	282		285		608		619	
4. Total macroscopic mutants, ex- perimental.....	50	8.5	69	10.06	502	38.4	123	8.7
5. Total macroscopic mutants, con- trol.....	2	0.34	0	0	2	0.19	5	0.80
6. Total <i>K</i> mutants, experimental...	1	0.102	1	0.14	414	31.1	21	1.4
7. Total <i>K</i> mutants, control.....	0	0	0	0	0	0	0	0
8. Others than <i>K</i> , experimental.....	49	7.6	68	9.91	88	6.6	102	7.2
9. Others than <i>K</i> , control.....	0	0	0	0	0	0	0	0
10. Total pigmented mutants, ex- perimental.....	13	2.2	11	1.6	3	0.22	9	0.63
11. Total pigmented mutants, con- trol.....	1	0.35	0	0	0	0	0	0
12. Average of lethal mutants, <i>per cent</i> .		61.4		72.3		73.3		69.3
13. Ratio								
Per cent germination, Exp.....		1.2		0.95		0.75		0.50
Per cent germination, Con.....								
14. Dose.....	112.81 × 10 ³		—		0.25-3.0		20.0-1800	
	(roentgens)				(joules/cm. ²)		(joules/cm. ²)	

In row 10 the pigmented mutants are shown separated from "other mutants." More are produced in x-ray experiments than in ultraviolet experiments. The mutants classed as "pigmented" include colonies with pink-red pigmented mycelium, green pigmented mycelium, and brown pigmented mycelium. Some of the pigmented mutants were very distinctive, for example red pigmented mutants were obtained in three x-ray experiments with the Shearer tube unit, but were not produced again although a number of experiments were made in an endeavour to do so. The same instability applies to the other pigmented mutants produced by x-ray irradiation.

The ratio of experimental to control germination (row 13) shows that germination is markedly affected at long ultraviolet and visible wave lengths

(ratio 0.5). A little effect on germination (ratio 0.75) is found with short ultraviolet, and no effect is apparent in x-ray irradiation (1.2 and 0.95 ratios).

In row 14 of the table the dose in roentgens required to produce 61.4 per cent lethal mutants with hard x-rays is shown to be 112.8×10^3 r. No measure of dose was made in soft x-ray experiments. In columns 3 and 4 the range of dose in joules/cm.² for ultraviolet and visible wave lengths is shown.

SUMMARY

1. Mutants produced by x-irradiation of fungal spores of *Chaetomium globosum* have been compared with those produced by ultraviolet irradiation.

2. The most striking difference between the mutants produced by x-irradiation and ultraviolet irradiation is the absence in x-ray experiments of the K mutant which is produced in large numbers at short ultraviolet wave lengths.

3. A comparison is made of the relation between x-ray dose and numbers of lethal mutants, and the relation between the short ultraviolet wave length 2804 dose and numbers of lethal mutants. Both are compared with theoretical curves for 1, 2, 5, and 8 quantum hits.

4. The production of lethal mutants by x-rays is shown to be consistent with the theoretical curve for five quantum hits on the sensitive spot of the spore, whereas the production of lethal mutants by the ultraviolet wave length 2804 Å.u. is consistent with two quantum hits.

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RESPIRATION OF THE TISSUES OF SOME INVERTEBRATES AND ITS INHIBITION BY CYANIDE*

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Present theories of the mechanisms of cell respiration have been developed primarily from investigations on only a few types of living material: yeast cells, sea urchin eggs, pigeon breast muscle, and mammalian tissues. The concepts derived from these studies may not be applicable to all animal tissues: not only may certain details in the picture be lacking, but the extent to which the conventional cytochrome-cytochrome oxidase scheme participates is in many cases as yet undefined. Although Keilin (1) more than twenty years ago confirmed the widespread occurrence of cytochrome in living cells, there are still many gaps in our knowledge of the distribution of this compound among the invertebrates. Also, the mere presence of cytochrome c in a cell gives no indication of the extent to which the cell is actually dependent upon the cytochrome system for its normal activity. The present study was undertaken to obtain comparative cell respiration measurements of certain representative invertebrate tissues and to determine how much of this oxygen consumption is dependent upon cyanide-sensitive mechanisms.

Experimental Technique

This investigation was made at the Bermuda Biological Station for Research during the fall and winter of 1947. Respiration measurements were obtained with a Warburg manometric outfit, using standard and micro flasks of about 17 cc. and 6 cc. volumes respectively. The rate of shaking was kept at 120 cycles per minute, with variation of the amplitude according to the nature of the material. The oxygen consumption was determined with the tissues in filtered sea water, with a 10 per cent KOH solution or a 10 per cent $\text{Ca}(\text{OH})_2$ suspension in the center wells to absorb CO_2 . Since the temperature of the water from which the animals were collected remained within a range of 27 to 17°C. during the course of the observations it was possible to make the observations at the relatively high level of 25°C.¹

Respiratory measurements on most of the tissues could be made without slicing

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¹During the summer it was difficult to keep certain animals in the laboratory for more than a few hours, but in early October when the temperature of the sea water supply dropped below 25°C. it was found that most of the specimens could be maintained in good condition for several days or even weeks.

the material since the structures themselves were within the limiting thickness. However, slices were made with a razor blade through the sponges and through the calcareous material along the sides of the gorgonian rods. It was possible to use rather thick slices of the sponges since their porous nature permitted rapid circulation of sea water. The branches of the purple sea fan were ground in a porcelain mortar, and the cellular suspension was decanted from the heavy skeletal debris. Barnacles were removed from their calcareous outer shells with as little injury as possible and measured without further treatment. The inert outer tunic was removed from the small tunicates, and with the large, simple tunicates, only the pharynx was studied.

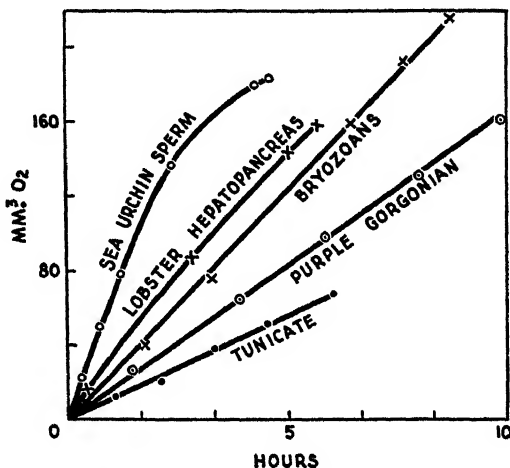


FIG. 1. Typical oxygen consumption curves for various invertebrate tissue preparations in Warburg manometer flasks in sea water. The bryozoans were intact; the tunicate had the inert tunic removed; lateral slices were made from along the sides of the gorgonian rod; the filamentous lobster hepatopancreas bundles were separated from each other.

Oxygen consumption determinations are expressed in the tables as QO_2 values ($\text{mm}^3\text{O}_2/\text{mg. dry weight}/\text{hr.}$). It was necessary to correct the gross dry weight measurements of those tissues which contained inert skeletal material, such as the sponges, coral animals, and barnacles. Samples of these tissues were dried and weighed, treated with trichloroacetic acid to fix the proteins, with 25 per cent HCl, washed, centrifuged, then redried and weighed. This eliminated the CaCO_3 and the soluble salts. The trichloroacetic acid was driven off during the drying period at 100°C . Further treatment with boiling 10 per cent KOH to dissolve the protein, washing, centrifuging, and a final weighing then gave the amount of inert siliceous or chitinous material. From these weights it was possible to compute the original dry weight of the tissue minus the skeletal material.

It is difficult to determine whether measurement of the oxygen consumption of a piece of isolated tissue is truly representative of the respiration of the tissue in its

normal environment. The course of oxygen uptake, however, may at least indicate whether there is a progressive change in the chemical systems in the tissue which are responsible for respiration. Fig. 1 shows typical oxygen consumption curves for several different types of tissue. Some specimens, such as the bryozoans and gorgonians, could be run for as long as 10 hours without decrease in rate. The bryozoans were probably uninjured since every animal is protected by enclosure in a small, transparent shell, partially open on one side to allow circulation of sea water. Each polyp in the gorgonian slices must have been cut, but the injury was apparently localized and did not affect the intact adjoining cells. Most tissues could be measured for at least a 2 to 3 hour period without appreciable falling-off in respiration. Certain types, such as the lobster tissues and some of the sponges, showed a continuously decreasing rate but this probably affected the measurement only slightly during the first

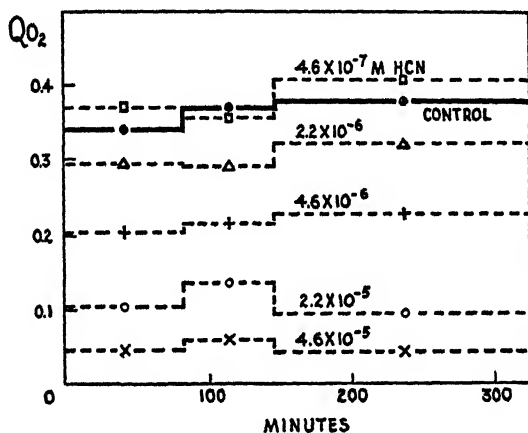


FIG. 2. Respiration of the epithelial eye covering or "cornea" of the squid in various concentrations of cyanide in sea water. The HCN tensions were maintained at constant levels by $\text{Ca}(\text{CN})_2 - \text{Ca}(\text{OH})_2$ center well mixtures.

hour or two. The only materials which were found to show an increase in the rate of oxygen consumption upon shaking were the sea urchin eggs (Fig. 6). This increase was probably due to development in the fertilized eggs, and to instability of the unfertilized ones.

In order to maintain constant concentrations of HCN in the sea water in the flasks, $\text{Ca}(\text{CN})_2 - \text{Ca}(\text{OH})_2$ solutions were used in the center wells (2). These "balanced" center well solutions absorb CO_2 without altering the tension of HCN in the air space in the flasks.³ It was found that HCN equilibrium between the center well mixture and the sea water is attained so rapidly that it is unnecessary to add cyanide directly to the sea water itself. For example, in the experiment represented in Fig. 2, ma-

³Although sea water becomes alkaline when its CO_2 is absorbed by the center well solution, the respiratory CO_2 was sufficient to prevent extensive shift in pH.

nometer readings were started 20 minutes after cyanide had been added to the filter paper in the center wells. The inhibition over the subsequent 5 hour period was constant, indicating that approximate HCN equilibrium was reached by the time the first reading was taken. This technique is convenient and it is also desirable because of the fact that nothing is added to the fluid containing the experimental material except the HCN gas from the center well mixture. A biological check of the HCN equilibrium levels of the center well solutions has shown that, even at $1 \times 10^{-6}M$, the concentration of HCN in the sea water is still at the theoretically correct level (3).

RESULTS

(a) *Respiratory Rate.*—The QO_2 values of the tissues studied are listed in Table I. Where there are ten or more determinations standard deviations are given. The figures range from extremes of 0.02 for the squid lens to 26 for sea urchin sperm, but the other values lie within a range of 0.4 to 3.0.

(b) *Respiration in Cyanide.*—Inhibition of respiration of tissues in various cyanide solutions is indicated by the values in Table II. Sea urchin sperm, squid gills, and lobster nerve and muscle are almost completely inhibited by low concentrations of cyanide, but many of the animals show only a partial sensitivity. The oxygen consumption of three tissues, the jellyfish, *Cassiopea*, the branchial tree of the sea cucumber, and the tunicates, was entirely unaffected by even 0.01 M HCN.

The constant rates of respiration in cyanide demonstrated by the curves in Fig. 2 are typical, although with a few kinds of animals high concentrations of HCN resulted in a gradual falling-off of oxygen consumption. This deviation from a constant rate was not usually evident until after more than 2 hours' exposure.

(c) *Concentration of HCN Necessary for 50 Per Cent Inhibition.*—Fig. 3 illustrates the typical spread of points obtained when observed oxygen uptake is plotted against the concentration of cyanide. Fig. 4 shows concentration-inhibition curves for a number of representative tissues. The concentration of cyanide necessary to cause 50 per cent inhibition of the sensitive portion of the respiration may range from $10^{-6}M$ (sea urchin sperm) to $10^{-3}M$ (sea fan). This widespread variation is indicated by the values in Table II for the equilibrium constant, K , which denotes the concentration of cyanide that produces 50 per cent inhibition of the sensitive portion of the respiration.

(d) *The Slope of the Inhibition Curve.*—A comparison of the curves shown in Fig. 4 is facilitated by applying the law of mass action to the data. The curves in Fig. 5 are derived by plotting the logarithm of the ratio of the inhibited to the uninhibited respiration against the log of the concentration of the inhibitor (4). (Since it is only the cyanide-sensitive part of the respiration which is being analyzed the portion unaffected by $10^{-3}M$ HCN was not considered.) Table III gives the slopes of the straight portions of the

TABLE I
Oxygen Consumption of Tissues of Bermuda Invertebrates

Animal	Tissue	No. of determinations	O ₂	
			Mean	Range
Sponges				
(<i>Tedania ignis</i>).....	Slices	6	-2.9	-2.5 to -3.7
(<i>Ircinia fasciculata</i>).....	"	5	-1.6	-1.2 to -2.1
(<i>Lissodendoryx isodictyalis</i>)..	"	4	-1.4	-1.1 to -1.6
(<i>Axinella rosacea</i>).....	"	2	-0.7	-0.6 to -0.8
(<i>Cinachyra cavernosa</i>).....	"	3	-0.6	-0.5 to -0.6
(<i>Dysidea crowshayi</i>).....	"	2	-0.6	-0.6 to -0.7
(<i>Terpios fugax</i>).....	"	1	-0.6	
(<i>Geodia gibberosa</i>).....	"	1	-0.6	
(<i>Tethya aurantia</i>).....	"	1	-0.5	
(<i>Spheciospongia sp.</i>).....	"	1	-0.4	
Coelenterates				
Purple gorgonian				
(<i>Plexaura flexuosa</i>).....	Slices	13	-3.0 ± .66	-2.4 to -4.5
Purple sea fan				
(<i>Gorgonia flabellum</i>).....	Suspension	2	-2.2	-2.1 to -2.3
Portuguese man-of-war				
(<i>Physalia pelagica</i>).....	Tentacles	3	-1.7	-1.1 to -2.2
Sea anemone				
(<i>Condylactis gigantea</i>)...	"	3	-0.8	-0.7 to -0.9
Jellyfish				
(<i>Pelagia cyanella</i>).....	Umbrella	2	-0.8	-0.8 to -0.9
(<i>Cassiopea frondosa</i>)....	"	18	-0.7 ± .17	-0.3 to -1.0
(<i>Cassiopea frondosa</i>)....	Tentacles	10	-0.6 ± .16	-0.4 to -1.0
Bryozoans				
Species of <i>Ectoprocta</i>	Entire	6	-2.1	-1.9 to -2.5
Echinoderms				
Sea urchin				
(<i>Tripneustes esculentus</i>)..	Sperm	3	-26	-18 to -41
" " ..	Eggs, (fertilized)	3	-0.6	-0.6 to -0.7
" " ..	Eggs, (unfertilized)	6	-0.1	-0.08 to -0.13
Sea cucumber				
(<i>Stichopus möbbii</i>).....	Intestine	12	-0.7 ± .10	-0.6 to -1.0
" "	Branchial tree	11	-0.6 ± .07	-0.5 to -0.7
Mollusks				
Squid				
(<i>Loligo pealei</i>).....	Gills	5	-1.8	-1.4 to -2.3
" "	Retina	3	-1.1	-0.9 to -1.4
" "	"Cornea"	4	-0.4	-0.4 to -0.5
" "	Lens	2	-0.02	-0.02 to -0.03
Black oyster				
(<i>Pedalion alata</i>).....	Gills	6	-1.3	-0.9 to -2.7

TABLE I—*Concluded*

Animal	Tissue	No. of deter- mina- tions	Q _{O₂}	
			Mean	Range
Crustaceans				
Goose neck barnacle (—)*.....	Entire	2	-3.0	-2.2 to -3.9
Lobster (<i>Panulirus argus</i>).....	Hepatopancreas	4	-3.0	-2.3 to -3.8
“ “	Leg nerve	5	-1.1	-0.9 to -1.4
“ “	Leg muscle	2	-1.0	-0.7 to -1.2
Tunicates				
Social tunicate (—).....	Entire animal except tunic	7	-1.3	-1.0 to -1.5
Simple tunicate (—).....	Pharynx	10	-1.1 ± .19	-0.8 to -1.4
Fish				
Red snapper (<i>Lutianus vivanus</i>).....	Retina	2	-2.1	-2.0 to -2.1

Q_{O₂} values are determined on a corrected dry weight basis (see text). Standard deviations are listed when there are ten or more determinations.

* Dashes indicate that scientific name was not determined.

curves. All the values approximate 1, except those for the relatively insensitive coelenterates. However, the data given in Table II but not plotted in Fig. 5 indicate that two of the sponges, the sea cucumber intestine, and the oyster gills would also give slopes of a value different than 1. The curve for the sea fan suspension may not represent a normal respiratory system, since the tissues were crushed and this disruption of the cells may have permitted spontaneous oxidation of endogenous substrate.

(e) *Experiments with Cassiopea*.—The jellyfish, *Cassiopea*, is one of the three forms listed in Table II whose respiration was completely unaffected by even high concentrations of HCN. Most of the oxygen consumption of this animal takes place in the cellular surface layer of either the tentacles or the dorsal or ventral coverings. The surface of the subumbrella is easily removed by cutting wedge-shaped pieces through the flat body and slicing off a millimeter or so with a razor blade. The denuded jelly layer had an oxygen consumption, on a dry weight basis, of only about one twenty-fifth the rate of the surface tissue. Results of experiments performed on the active surface cells are recorded in Table IV. Neither HCN nor 10⁻³M NaN₃ produced inhibition of respiration within a 2 hour period, although the same concentration of the latter agent caused an 87 per cent depression of the oxygen con-

TABLE II
Respiration of Tissues of Bermuda Marine Animals in Cyanide

Animal	Tissue	No. of deter- mina- tions	O ₂ consumption in HCN (Per cent control)				K
			10 ⁻² M	10 ⁻³ M	10 ⁻⁴ M	10 ⁻⁵ M	
Sponges							
(<i>Tedania ignis</i>)	Slices	18	27	30	34	56	10 ^{-5.2}
(<i>Ircinia fasciculata</i>)	"	18	21	24	28	56	10 ^{-5.6}
(<i>Dysidea crawshayi</i>)	"	12	26	27	30	48	10 ^{-5.5}
(<i>Axinella rosacea</i>)	"	6	47	59	72	84	10 ^{-4.2}
(<i>Cinachya cavernosa</i>)	"	6	29	49	63	80	10 ^{-4.0}
(<i>Haliclona viridis</i>)	"	18	28	31	37	53	10 ^{-5.4}
(<i>Chondrilla nucula</i>)	"	18	26	27	30	59	10 ^{-5.1}
(<i>Spirastrella coccinea</i>)	"	1		77			
(<i>Lissodendoryx isodictyalis</i>)	"	4		31			
(<i>Tethya aurantia</i>)	"	1		59			
(<i>Terpios fugax</i>)	"	1		45			
(<i>Spherospongia</i> sp.)	"	1		68			
(<i>Geodia gibberosa</i>)	"	1		43			
(<i>Leuconia barbata</i>)	"	1		19			
Coelenterates							
Sea anemone							
(<i>Condylactis gigantea</i>)	Tentacles	18	37	50	63	79	10 ^{-4.2}
Purple gorgonian							
(<i>Plexaura flexuosa</i>)	Slices	22	39	57	81	97	10 ^{-3.5}
Brown gorgonian							
(<i>Pseudoplexaura crassa</i>)	"	22	37	56	75	96	10 ^{-3.7}
Purple sea fan							
(<i>Gorgonia flabellum</i>)	Suspension	23	32	64	99	100	10 ^{-3.0}
Jellyfish							
(<i>Cassiopea frondosa</i>)	Subumbrella	25	100	100	100	100	
Portuguese man-of-war							
(<i>Physalia cyanella</i>)	Tentacles	12	12	16	46	87	10 ^{-4.2}
Bryozoans							
Species of <i>Ectoprocta</i>							
(—)	Entire	12	20	21	29	66	10 ^{-4.9}
Echinoderms							
Sea cucumber							
(<i>Stichopus mōbiū</i>)	Intestine	13	36	42	58	80	10 ^{-4.4}
" "	Respiratory tree	16	100	100	100	100	
Sea urchin							
(<i>Tripleneustes esculentus</i>)	Eggs, (unferti- lized)	20	27	27	37	72	10 ^{-4.8}
" "	Eggs, (ferti- lized)	16	12	12	13	25	10 ^{-5.5}
" "	Sperm	15	3	3	4	7	10 ^{-6.1}

TABLE II—*Concluded*

Animal	Tissue	No. of deter- mina- tions	O ₂ consumption in HCN (Per cent control)				K
			10 ⁻² M	10 ⁻³ M	10 ⁻⁴ M	10 ⁻⁵ M	
Mollusks							
Black oyster							
(<i>Pedalion alata</i>).....	Gills	12	24	30	38	46	10 ^{-5.3}
" "	Mantle	12	20	20	21	33	10 ^{-5.7}
Arca							
(<i>Arca noae</i>).....	Gills	6	67	73	78	84	10 ^{-5.0}
Squid							
(<i>Loligo pealei</i>).....	Gills	17	6	6	10	52	10 ^{-5.0}
" "	Retina	15	22	24	28	56	10 ^{-5.0}
" "	Lens	10	18	20	27	61	10 ^{-5.0}
" "	"Cornea"	11	20	20	20	48	10 ^{-5.2}
Crustaceans							
Lobster							
(<i>Panulirus argus</i>).....	Leg nerve	12	3	4	6	19	10 ^{-5.3}
" "	Leg muscle	6	4	5	14	30	10 ^{-5.5}
" "	Hepatopancreas	12	20	21	30	78	10 ^{-4.7}
Goose neck barnacle							
(—).....	Entire	12	12	16	24	56	10 ^{-5.0}
Tunicates							
Large simple tunicate							
(—).....	Pharynx	15	100	100	100	100	
Small social tunicate							
(—).....	Entire animal except tunic	12	100	100	100	100	
Fish							
Red snapper							
(<i>Lutjanus vivanus</i>).....	Retina	9	7	9	22	84	10 ^{-4.5}

The *K* values in the last column represent the HCN concentration which produces 50 per cent inhibition of the cyanide-sensitive portion of the respiration.

sumption of squid gills. After 3 hours in 10⁻²M HCN, the jellyfish subumbrellar tissue began to deteriorate and the oxygen consumption declined, but the intact animals could be kept in 10⁻³M HCN in sea water for at least 48 hours without apparent injury. The cyanide-sea water for this experiment was changed frequently to maintain adequate oxygen tension and also to assure that the tissues did not deplete the cyanide by some process of detoxication. Survival of muscular movement and active nerve conduction was shown by the regular periodic contractions of the animal.

After the *Cassiopea* subumbrellar tissue had been warmed to 50°C. for 5 minutes, the subsequent respiration was about one-fifth normal, and when

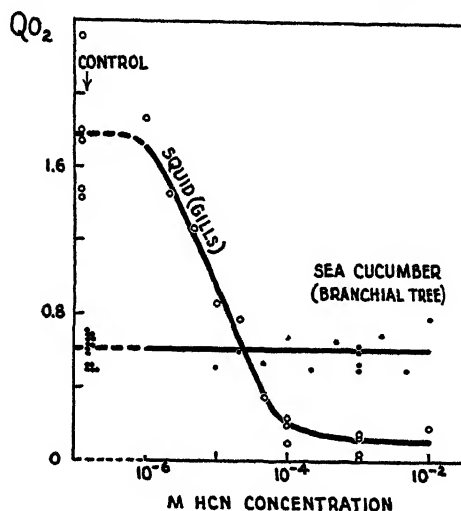


FIG. 3. Representative cyanide inhibition data for a sensitive tissue (squid gills), and one which is unaffected by HCN (sea cucumber branchial tree).

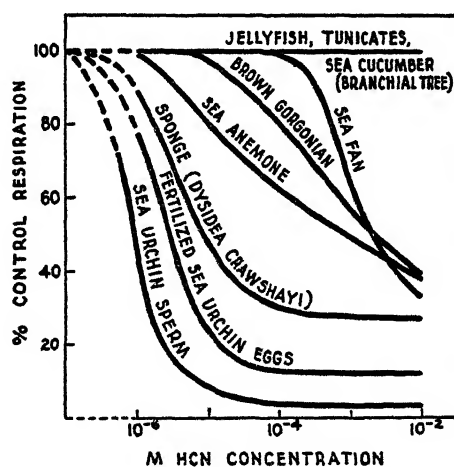


FIG. 4. Variation in oxygen consumption with concentration of cyanide for nine types of marine invertebrate material. Species names are listed in Table II.

heated to 65°C. there was a further reduction to one-fifteenth that of the control, indicating that a heat-sensitive enzyme system is involved. Drying the tissue for 7 hours at only slightly elevated temperature also resulted in

almost complete loss of oxygen uptake. Crushing the cells in a glass tissue grinder lowered the respiration rate about 50 per cent.

TABLE III
Slope Values for the Curves Shown in Fig. 5

Curve	Tissue	Slope
1	Red snapper retina	1.22
2	Rat spleen (5)	1.26
3	Rat retina (6)	1.03
4	Rabbit lens (7)	1.05
5	Sea urchin sperm	0.88
6	Fertilized sea urchin eggs	1.16
7	Squid gills	0.98
8	Sponge (<i>Dysidea crawshayi</i>)	1.08
9	Sea anemone tentacles	0.46
10	Brown gorgonian	0.76
11	Sea fan	1.93 and 1.31

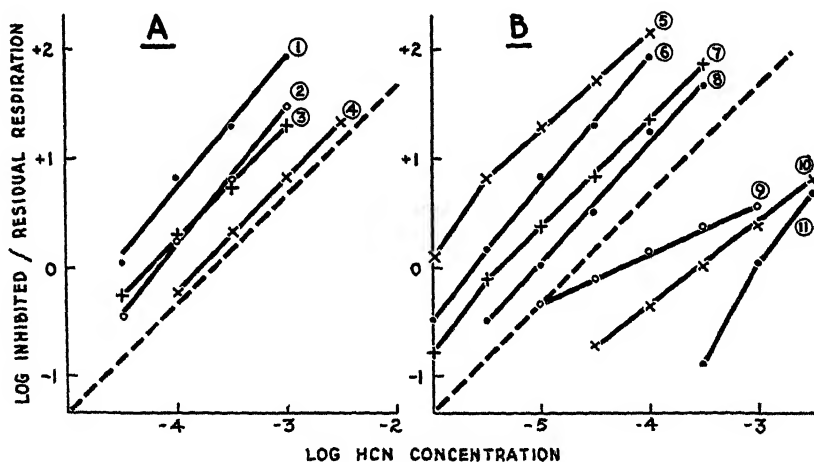


FIG. 5. Log ratio inhibited respiration/residual respiration against log cyanide concentration for vertebrate tissues (A), and invertebrate tissues represented in Fig. 4 (B). Types of tissues are listed in Table III. All values are corrected by subtracting the cyanide-resistant respiration at $10^{-2}M$ HCN.

Exposure to H_2S -saturated sea water for 10 minutes resulted in a considerable rise in oxygen consumption, but this may have been due to oxidation of the adsorbed sulfur during the subsequent measurement period. Intracellular pH factors are important in determining whether the sulfide will combine effectively with heavy metal, and the negative results obtained do

TABLE IV

Oxygen Consumption of the Subumbrellar Surface of the Jellyfish, Cassiopea frondosa, with Various Experimental Treatments

Treatment	QO ₂
Control; under surface of umbrella.....	-0.70
Control; jelly layer.....	-0.025
Unaffected	
HCN, 10 ⁻² M, 2 hrs.....	-0.75
HCN, 10 ⁻² M, 45 hrs. (unchanged).....	—
NaN ₃ , 10 ⁻² M.....	-0.61
NaF, 10 ⁻² M.....	-0.65
5 min. at 35-37°C.....	-0.77
Inhibited	
5 min. at 50-51°C.....	-0.15
5 min. at 65-66°C.....	-0.05
Dried 7 hrs., at about 35°C.....	-0.06
Ground in glass tissue grinder.....	-0.33
Stimulated	
H ₂ S-saturated sea water, 10 min.....	-2.9
Methylene blue, 10 ⁻³ M.....	-2.0
Toluylene blue, 10 ⁻³ M.....	-2.7

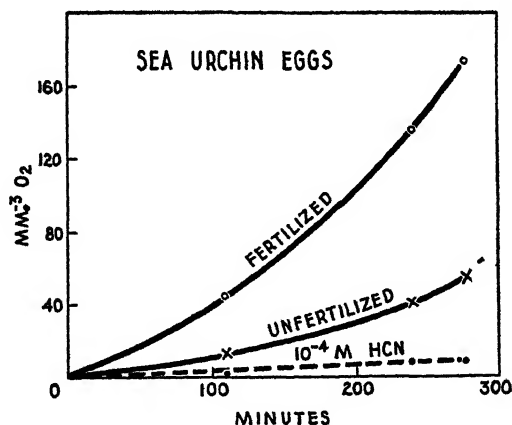


FIG. 6. Oxygen consumption of a suspension of sea urchin eggs (*Tripneustes esculentus*) before and after fertilization, and in 10⁻⁴M HCN. The curves for both fertilized and unfertilized eggs in cyanide are identical.

not necessarily mean that no heavy metal is active. Methylene blue and toluylene blue were also tried. They caused some acceleration of oxygen uptake.

(f) *The Effect of Cyanide on Sea Urchin Eggs.*—The oxygen uptake of the eggs of the white sea urchin, *Tripneustes esculentus*, is represented by the curves in Fig. 6. There is a three- to fivefold stimulation of respiration on fertilization, which is comparable to that of *Arbacia* and other sea urchins. The effect of various concentrations of cyanide on the oxygen consumption of the eggs is listed in Table II. Fig. 6 shows that $10^{-4}M$ HCN almost completely inhibits the oxygen consumption of both unfertilized and fertilized eggs and that the residual respiration of both in cyanide is identical. Complete inhibition of cell division occurs at a concentration of slightly less than $10^{-4}M$ HCN. The respiration of the fertilized egg in this solution is reduced to about 40 per cent of the control level.

DISCUSSION

A comparison of the respiration of tissues from marine invertebrates at 25°C. with that of vertebrate tissues at the same temperature shows that in some cases the former may approximate the latter (Fig. 7). Although certain of the lower animals are metabolically sluggish there are others that can apparently function as actively as the higher forms if the temperature level is comparable. The cyanide sensitivity studies show that heavy metal systems participate in the cellular respiration of most primitive forms. If such animals as the bryozoans have maintained as much physiologic constancy during geologic history as they have morphologic uniformity, then it may be said speculatively at least that the use of heavy metal electron transfer systems in cell respiration may have been one of the first steps in the evolutionary development of living matter.

A quantitative study of the inhibition of cellular respiration by cyanide can provide three types of information: (1) the magnitudes of the cyanide-sensitive and insensitive portions; (2) the concentration of HCN required to produce 50 per cent inhibition (the equilibrium constant K value); and (3) the slope of the inhibition curve.

Although depression of oxygen consumption by low concentrations of cyanide is indicative of heavy metal catalysis, it is not conclusive evidence of cytochrome oxidase activity. Cyanide may inactivate copper and other heavy metals as well as iron, and possibly other mechanisms than the cytochrome system are active in certain animals. The cyanide experiments are valuable because this agent penetrates uninjured cells and indicates how much of the normal respiration is mediated by sensitive systems.

The cyanide-resistant respiration is plotted in Fig. 7 in actual QO_2 values rather than as percentage inhibition, since Commoner (10) has shown that, for several tissues at least, the cyanide-resistant portion is constant but the magnitude of the sensitive portion may increase greatly upon the addition of substrate. It may be noted in this connection that the vertebrate tissues

represented in Fig. 7 were in saline solution containing glucose, whereas the invertebrates had endogenous substrate only.

Several hypotheses have been suggested to account for the cyanide-insensitive portion of cellular respiration: (a) Gourévitch (11) found that those tissues which have a high riboflavin content are most insensitive, perhaps because flavoprotein can be directly oxidized by molecular oxygen. (b) Certain stages of fat and protein oxidation are also independent of the cytochrome system (10), and are therefore not sensitive to cyanide. (c) Since cytochromes

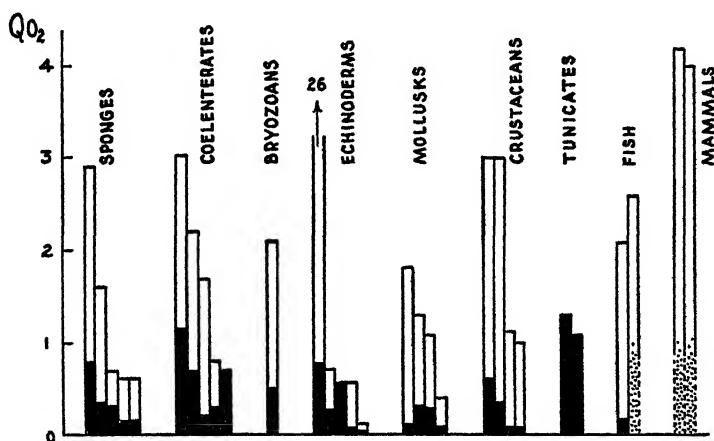


FIG. 7. A graphic comparison of the respiration and cyanide sensitivity of invertebrate and vertebrate tissues (25°C.). The solid portions of the columns indicate residual respiration in 0.01 M HCN. The last three columns represent oxygen consumption of bass brain (8), rat liver (9), and rat brain (8) respectively. The lower parts of these columns are left indeterminate by dotting since measurements of the cyanide sensitivities at this temperature are not available. Tissues represented by the other columns may be identified from the values in Tables I and II.

a and b do not combine with cyanide a certain amount of oxidation may proceed even in the presence of the inhibitor (12). (d) Another explanation is that although the addition of cyanide to cytochrome oxidase lowers the oxidation-reduction potential, the resulting compound may still be capable of oxidizing flavoprotein (13).

Complete insensitivity of the respiration of a living tissue to cyanide has seldom been observed. *Paramecium caudatum* is the classic example of a "cyanide-insensitive" organism, but Pace (14) has recently demonstrated that it is only the starved animals that are uninhibited: normal specimens are 42 to 66 per cent depressed by 10^{-3} M HCN, depending upon their age.

It is perhaps more than coincidental that two of the three tissues found

in the present study to be completely cyanide-insensitive have been reported to contain a high concentration of vanadium. Vanadium does not combine with cyanide (15) and a catalytic system dependent upon this metal for electron transfer should be unaffected by HCN. Vanadium is almost absent from sea water and it was possible to determine only a trace of it in the residue from 200 liters (16). However, tunicates have been known for a long time to contain this metal (17-19), and the same species of sea cucumber used in the present experiments was found to have a vanadium content of 0.12 per cent of the dry weight when collected at the Tortugas (20). Spectrographic analysis of a number of other kinds of biological materials has shown that vanadium is usually absent or if it is present it is found in extremely low concentration (21). This scarcity of the metal suggests that the correlation of high vanadium content and cyanide insensitivity in the tunicates and sea cucumbers is possibly more than coincidental. (Although the increased oxidation of phospholipids by liver suspension containing added sodium metavanadate was inhibited by cyanide (22), this may have been due to involvement of other heavy metal systems.) The possibility of vanadium participating in the cellular respiration of these cyanide-insensitive forms as an electron-transfer catalyst will be more thoroughly investigated.

The great range and the thousandfold difference in the extremes of the K values listed in Table II may be indicative of some as yet unknown variable in the cellular respiration system. An adequate explanation of this difference in the sensitivity of various animals to HCN is lacking at present.

When inhibition data are plotted logarithmically as in Fig. 5 a regular variation of respiration with the concentration of the inhibitor results in a straight line. If the slope of the line is 1 it is an indication that one mole of the inhibitor is combined with one mole of the enzyme (4). Since most of the curves do give slopes approximating 1 it is evident that a 1-to-1 relationship of enzyme and inhibitor is the usual condition. It may be significant that in all the anomalous cases a high proportion of the respiration is unaffected by cyanide, indicating probably the participation of another type of system. Deviations from a slope of 1 may result if there is inhibition of more than one metabolic step, or if the kinetics of respiration do not depend only upon the concentration of the enzyme (23). Fisher (24) lists slope values from 0.52 to 1.6 for data obtained from various types of material by several investigators; difficulties in controlling cyanide concentrations during measurement periods may have contributed to this wide variation.

As shown in Fig. 6 and Table II the respiration of the unfertilized egg of the sea urchin, *Tripneustes*, is almost completely inhibited by 10^{-4}M HCN. This is comparable to the situation found in *Arbacia*, where, although it had been supposed that the unfertilized egg was insensitive to cyanide, improved techniques have shown that the respiration is at least 60 per cent depressed by 10^{-4}M HCN (25).

SUMMARY

A study of the metabolism of Bermuda marine invertebrates at 25°C. shows that the respiratory rates of many of the tissues approximate those of vertebrate tissues at the same temperature. There is no apparent correlation between respiratory rate and phylogenetic development: tissues from some of the simpler forms use as much oxygen per unit weight as those from certain of the more highly developed animals.

Cyanide inhibition experiments reveal a great variation in the amount of oxygen consumption which is dependent upon sensitive heavy metal systems. Three types of tissues, the jellyfish *Cassiopea frondosa*, the branchial tree of the sea cucumber, *Stichopus möbii*, and two kinds of tunicates, were completely unaffected by even 10^{-2}M HCN. Other tissues such as sea urchin sperm, squid gills, and lobster nerve and muscle were almost completely inhibited by much lower concentrations. Most of the materials retained 20 to 40 per cent of the normal respiratory rate in 10^{-3}M HCN. The possibility that vanadium may play a part in the oxidation-reduction systems of the completely resistant animals is discussed.

There is a thousandfold variation in the concentration of cyanide required to produce 50 per cent inhibition of respiration in the different tissues. Sea urchin sperm is 50 per cent inhibited by 10^{-6}M HCN: the sea fan requires 10^{-8}M for the same effect. Other tissues lie at intermediate points.

When the logarithm of the ratio of the inhibited to the uninhibited respiration is plotted against the concentration of cyanide the resulting line has a slope which in most cases approximates 1. This indicates that one mole of enzyme ordinarily combines with one mole of inhibitor.

Eggs of the sea urchin, *Tripneustes esculentus*, show a three- to fivefold increase in the rate of oxygen uptake on fertilization. The respiration of both the fertilized and unfertilized eggs is almost entirely inhibited by 10^{-4}M HCN. Cell division in the fertilized eggs is blocked by somewhat less than 10^{-5}M cyanide, a concentration which reduces respiration to 40 per cent of the normal level.

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CORRELATION OF THE AQUARIUM GOLDFISH TOXICITIES OF SOME PHENOLS, QUINONES, AND OTHER BENZENE DERIVATIVES WITH THEIR INHIBITION OF AUTOOXIDATIVE REACTIONS

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INTRODUCTION

In the course of some investigations on the effects of certain chemicals on goldfish, it was noted that the toxicity of hydroquinone and of tertiary butyl catechol in the aquarium water is about a hundred times higher than that of phenol; while their toxicity on injection into mammals and into fish is at most twice as high. Tertiary butyl catechol and hydroquinone show a high toxicity for the small entomostracan *Daphnia magna*, forty and a hundred times that of phenol. This suggests that the aquarium toxicity of these poisons is qualitatively different from that of phenol and from their action when administered to mammals or injected into fish. Hydroquinone and analogous substances are noted for their powerful inhibitory action on autocatalytic "chain reactions," such as the oxidative changes that lead to rancidity in fat; the "aging" of rubber; the polymerization of rubber; and the "cracking" of gasoline. The high "toxicity" of hydroquinone to these reactions is illustrated by the observation of Moureau and Dufraisse (1927) that one molecule of hydroquinone prevents the oxidation of 40,000 molecules of acrolein. The effective quantities are so minute that the anticatalytic actions are presumably exerted on some critical points in the autocatalytic chain, perhaps by the resonance of the quinone structure, involving oxidation or reduction in some cases and not in others (Milas, 1929, 1932). This could have analogies, although perhaps only superficial, with biologic processes. If so, there should be correlation between the chemical reactivity of these substances *in vitro* and their biologic reactivity, as expressed for instance in their aquarium toxicity. Such correlation would not prove that the particular inhibition of autocatalytic activity is the direct cause of the biologic response; they could both be manifestations of an underlying property of the substance, such as intramolecular mobility. The correlation would, however, point out directions for further inquiry along these lines. Correlation of the biologic action with the chemical reactivity would be a step nearer to the essence of the action, than attempts at correlation with the chemical constitution, which after all must operate by modifying the chemical reactivity of the substance.

The following experiments are the initial part of an exploration of the practical usefulness and limitations of this line of inquiry, by attempting the correlation of a variety of such autocatalytic reactions with fish toxicity on the one hand, and with their oxidation potential on the other. The aquarium method offers the special advantage that the relatively large volume of the solution, which is several hundred times that of the fish, would keep the concentration in the blood and tissues of the animal uniform and constant for the entire duration of the experiment, provided that the substance is sufficiently stable in aqueous solution; and that the substance is absorbed from the aquarium more rapidly than it is destroyed in a 2 day exposure of the fish. It should therefore give more absolute values than are obtained when the concentration in the animal declines continuously by excretion and destruction, after the drug is injected. It should be remembered, however, that the aquarium toxicity may depend on local actions, especially on the gills, and need not be identical with the systemic toxicity.

Methods

Goldfish, generally 3.5 to 6.5 cm. in length (exclusive of the tail fin), weighing 3 to 10 gm. were placed singly in glass aquaria filled with 2 liters of Cleveland city water which had stood for a day in the same room as the storage aquarium, so as to guard the fish against sudden change of temperature. The agent under investigation was added and the symptoms observed during the 1st day and the fatality noted to the end of the 2nd or 3rd day and sometimes later. The solution was renewed daily if the substance was liable to change. Death after 48 hours was so exceptional that this period was selected as the time for the fatality calculation. The "approximate fatal concentration" was taken as the geometric mean between the largest concentration that was survived for 48 hours, and the smallest concentration that was fatal in this time for practically all fish.

Since the aquarium toxicity of the different substances spreads so widely (the most toxic in the present series being about 2500 times more potent than the least toxic) it sufficed for the purpose of preliminary exploration to determine the fatal concentration with a rather wide margin, so that the lowest fatal concentration was up to 4 times higher than the highest non-fatal concentration. This wide spread is well beyond the limit of ordinary biologic variation, so that it is not necessary to use many animals. The effects practically always fall smoothly into series with the dosage, even when only one animal is used at each dose level. It is also well beyond the effects of variations of the room temperature of the aquarium water (18–25°C.).

The concentration is expressed as parts per million (p.p.m.), corresponding to milligrams per liter, or 2 mg. per aquarium, or 250 mg. per kg. of fish of the average weight of 8 gm. The p.p.m. concentration is preferred for convenience in calculating the fatal concentration. The difference from the molecular concentration would generally fall within the spacing of the dosage.

The *geometric mean* is the square root of the product of two numbers. For instance, if the fish survive 25 p.p.m. but die at 100 p.p.m., the geometric mean of survival concentration is $\sqrt{25 \times 100} = 50$ p.p.m. The relationship be-

TABLE I
Correlations with Aquarium Fish Toxicities
(Arranged in descending sequence of toxicities)

Substance	Data								Ranking					
	Fish toxicity			Oxidation potential (E_0) (Fieser)	Photographic reduction potential (Lowe)	Gasoline induction period (Lowry, <i>et al.</i>)	Rubber antioxidants (Kehc)	Rubber accelerators (Kehc)	Oxidation potential	Photographic reduction potential	Oil stabilization	Gasoline induction period	Rubber antioxidants	
	Approximate fatal concentration	Approximation	No. of animals used											
	<i>p.p.m.</i>	X:		<i>volts</i>										
Hydroquinone.....	0.287	1.15	27	0.631	1	135				3	2	1	5½	
<i>p</i> -Methylaminophenol (metol, elon).....	0.5	2	4	0.603	20					1	7			2
2,2,4-Trimethyl (α -phenyl- isopropyl)1,2-dihydro- quinoline (akanol).....	1.8	1.8	4				0.75							
<i>p</i> -Aminophenol.....	2	2	4	0.673	6	1470				4	4		2	
2-Mercaptobenzothiazole (captax).....	2	2	4	0.785				1		7				
Hydroquinone monobenzyl ether (agerite alba).....	2.5	1.67	5				>10							6
<i>n</i> -Phenyl- β -naphthyl- amine (agerite powder)....	4.4	1.36	6				1							3½
Aniline hydrochloride.....	5.5	1.8	3	1.135		60			13				5½	
<i>p</i> -Isopropoxy diphenyl- amine (iso).....	5.7	1.7	3				0.6							1
<i>p</i> -Phenylenediamine	5.74	1.74	6	0.710	0.4	765				5	1		4	
Pyrocatechol.....	14	1.4	3	0.742	7	1890				6	5		2½	1
Pyrogallol.....	18	1.8	5	0.609	16	1440				2	6		2½	3
<i>p</i> -Hydroxyphenylglycine....	20	2	2	0.833	1.6					9	3			
Phenol ..	28.9	1.15	8	1.089		60				12		6	8½	
4'-Chloro-2,5-dihydroxy diphenyl sulfone.....	35	1.15	4				1.5							5
4,5-dimethyl-2-mercapto- thiazole.....	56	1.4	3					0.85						
Resorcinol.....	57.4	1.7	3	1.043		135			11		5	5½		
<i>p</i> -Octyl diphenylamine.....	>40	—	4				1							3½
2,4-Diaminophenol hydro- chloride (amidol).....	80	2	3		30-40					8				
Hydrquinone mono- methyl ether.....	200	2	4	0.848					10					
Phloroglucinol.....	630	1.6	4	0.799		75			8		4	7		
<i>P</i> (probability of accidental correlation).....										0.09	0.20	0.20	0.265	0.39

tween these values is such that the smaller number multiplied by a constant, and the larger number divided by the same constant, equal the geometric mean. This constant or "spread" factor, expressed as $(\times \div)$ indicates the degree of approximation. It may be obtained by dividing the geometric mean into the

larger number. In the above example, the spread factor is $100 \div 50 = 2$; and the approximate fatal concentration would be expressed as 50 p.p.m. ($\times \div 2$).

The relative aquarium toxicities and the inhibitory effects of the substances on various autooxidative reactions were compared according to the rank order method of Friedman (1937), *viz.*

$$\chi^2_R = \frac{12}{np(p+1)} \sum (\text{rank totals})^2 - 3n(p+1)$$

Where n is the number of series being compared (*i.e.*, two in this investigation); p is the number of items being ranked in each series.

TABLE II
Rank Correlations with Oxidation Potential
(Arranged in ascending sequence of E_o)

Substance	Ranking				
	Oxidation potential	Aquarium fish toxicity	Photographic reduction potential	Oil stabilization	Gasoline induction period
<i>p</i> -Methylaminophenol (metol, elon)	0.603	2		7	
Pyrogallol	0.609	8	2½	6	3
Hydroquinone	0.631	1	1	2	5
<i>p</i> -Aminophenol	0.637	3½		4	2
<i>p</i> -Phenylenediamine	0.710	6		1	4
Pyrocatechol	0.742	7	2½	5	1
2-Mercaptobenzothiazole	0.785	3½			
Phloroglucinol	0.799	13	4		7
<i>p</i> -Hydroxyphenylglycine	0.833	9		3	
Hydroquinone monomethyl ether	0.848	12			
Resorcinol	1.043	11	5		6
Phenol	1.089	10	6		9
Aniline hydrochloride	1.135	5			8
<i>P</i> (probability of accidental correlation) . . .		0.09	0.153	0.10	0.099

This method¹ provides a measure of the probability, P , of obtaining through chance alone an observed degree of agreement between the two (or more) series of rankings. A small value of P indicates that factors other than chance are probably operating and implies a relationship between the two methods under

¹ I am greatly indebted to Dr. G. F. Badger, not only for suggesting this method of analysis, but also for working out the computations and advising on their significance. Dr. José J. Estable assisted in many of the later experiments.

consideration. The values of χ^2_R in terms of P are obtainable from the tables of Pearson (1930).

The data for the aquarium toxicity and various autocatalytic reactions are shown in Table I, as are also their rank order and their P (the probability that the rank correlation could occur by chance). Table II gives the correlation of their ranking with the oxidation potential. The rank sequence in these tables shows the general correspondence and the outstanding exceptions.

Correlations with Oxidation Potential

Fieser (1930) published determinations of the "critical oxidation potentials" (E_c), the potential (in volts) at which the rate of oxidation of the reductant of an oxidation-reduction system becomes so small as to be just detectable. Data on both this E_c and the aquarium goldfish toxicity (FT) are now available for thirteen benzene derivatives. In nine, or 70 per cent of these, the two properties differ in rank by $\frac{1}{6}$ or less (2 positions) of the total positions. Three, or 23 per cent differ by more than $\frac{1}{3}$ (4 positions), namely aniline, E_c 13, FT 5, difference + 8; phloroglucinol, E_c 8, FT 13, difference - 5; pyrogallol, E_c 2, FT 8, difference - 6. Presumably some special factor enters for these substances. With pyrogallol and perhaps also with phloroglucinol, the toxicity would be lowered by oxidation of the aqueous solution in the aquarium. The relatively high toxicity of aniline may be due to a different mechanism of action, such as the formation of methemoglobin. The mercaptan benzothiazole (E_c 7, FT $3\frac{1}{2}$) also introduces other chemical groups which are likely to have different actions.

The probability, P , for the entire series is 0.09, which is more than suggestive. If aniline is omitted, P of the remaining twelve substances becomes 0.06, which is quite good.

Correlation with Photographic Developers

Lowe (1939) grades the effectiveness of substances commonly used for this purpose by their "reduction potential," defined by the amount of potassium bromide that must be added to the developer to produce a specified decrease in the activity of the chemical in question.

Comparing the photographic reduction potential with the 8 compounds for which both data are available, five (62 per cent) differ by less than $\frac{1}{6}$ of the total positions ($1\frac{1}{3}$ places) in rank. Three (38 per cent) differ by more than $\frac{1}{3}$ ($2\frac{2}{3}$ places). The P of the entire series is 0.20, which is at least suggestive.

Comparing the photographic reduction potential with the critical oxidation potential of the seven substances for which both data are available, three (43 per cent) differ by less than $\frac{1}{6}$ ($1\frac{1}{3}$ places) in rank. Two (29 per cent) differ by more than $\frac{1}{3}$ ($2\frac{2}{3}$ places). Excluding these two gives P 0.109. P of the entire series is 0.153, rather better than the correlation with fish toxicity.

Stabilization of Fatty Oils

Rancidity of fats is due to the autooxidation of a small amount of their unsaturated fatty acids. It involves the production of peroxides. There is a considerable "lag" or "induction period," during which the change is very slow; but when once started it proceeds progressively faster, by autocatalytic chain reactions. The long lag of natural fats is due to their content of antioxidant substances, not well identified, but some, at least, related to tocopherol (Olcott *et al.*, 1936). The lag is overcome by the gradual destruction of these antioxidants by peroxidases (Boehm and Williams, 1945). The autooxidation may be very effectively inhibited by the addition of a variety of "stabilizing agents" (Jamieson, p. 26), especially phenolic substances and aromatic amines. The efficiency of these antioxidants can be rated quantitatively by various methods. Half a dozen series of determinations are available totaling nearly thirty substances, with fairly concordant ranking, although different criteria were used. These lists include six of the phenolic substances for which we have aquarium fish toxicity and oxidation potential data.

Correlation of the ranking of the fish toxicity with that of oil stabilization shows that of the six phenols, five (83 per cent) differ in rank by $\frac{1}{6}$ (1 position) or less of the total positions. None differs by $\frac{1}{3}$ (2 positions) or more. The P is 0.20 which is suggestive.

Correlation of the oxidation potential with the oil stabilization rankings shows that five (83 per cent) differ in rank by $\frac{1}{6}$ (1 position) or less; none by $\frac{1}{3}$ (2 positions) or more. P is 0.10 which is more than suggestive.

It appears therefore, that there is correlation between the aquarium fish toxicity and the inhibition of the autocatalytic oxidation of oils; and an even higher correlation between this and the oxidation potential.

Correlation with Inhibition in "Cracking" of Gasoline

Lowry *et al.* (1933) correlated the critical oxidation potential with the effectiveness of a number of agents in breaking the chain reactions of this thermal decomposition process by an accelerated oxidation test, and found a considerable agreement in ranking, with a few notable exceptions which could be explained by the fact that the higher temperature of their test probably produced changes in the inhibitors, especially phloroglucinol and hydroquinone.

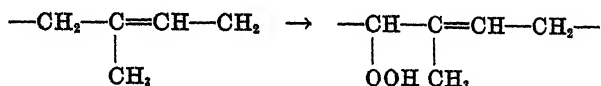
Comparing the gasoline induction period and the aquarium fish toxicity of the nine compounds for which both data are available, three (33 per cent) differ by $\frac{1}{6}$ ($1\frac{1}{2}$ positions) or less in rank. Four (44 per cent) differ by $\frac{1}{3}$ (3 positions) or more. The P is 0.265, a rather poor correlation. Excluding the three extremes, aniline, hydroquinone, and pyrocatechol, improves P to 0.112.

The correlation of the gasoline induction period with the oxidation potential is much better. Of the nine substances, seven (78 per cent) differ by less

than $1/6$ ($1\frac{1}{2}$ positions or less; two (22 per cent) differ by $1/3$ (3 positions) or more. P is 0.099, which is more than suggestive. Excluding pyrocatechol and hydroquinone reduces P to 0.078.

Antioxidants ("Age Resisters") of Rubber Industry

The manufacture of rubber involves chain reactions at several points. The deterioration of the distinctive physical characteristics of rubber is due to oxidation, probably by the formation of an unstable peroxide from the isoprene units (Fieser and Fieser):



This change can be retarded by antioxidant catalysts, such as aromatic amines, phenols, and quinones, added in the ratio of about $\frac{1}{2}$ to 1 per cent. These stabilizers of rubber are also effective for the stabilization of fats (Lea, p. 173). The B. F. Goodrich Chemical Company, kindly provided nine of these substances for comparison with their aquarium goldfish toxicity. Many were found poorly soluble in water, but fatal concentrations of the majority were obtained by first dissolving them in a little ethyl alcohol, well below its toxic concentration. Three of the substances were not fatal in saturated solution and were not counted, as their concentration was not known, but they were not important for the present purpose, as the successful tests included closely related substances. This left six substances shown in Table I. Four are highly toxic (1.8 to 5.7 p.p.m.): akanol, agerite alba, agerite powder, and iso. The other two are considerably toxic, 35 to >40 p.p.m. It appears that the age-resistant antioxidants generally have a high aquarium toxicity.

Quantitative data on the potency of these substances on age-resisting of rubber are not available in the literature. The review of Jacobs (1933) contains only one of the substances (*n*-phenyl- β -naphthylamine; agerite powder; neozone D, M.C.), and this ranks very differently according to conditions. Mr. Kehe of the Goodrich Company kindly gave the personal estimates shown in Table I with the caution that "these ratings are not very accurate and the differences indicated may be smaller or larger depending on a great number of other conditions." These estimates give a very poor correlation with the aquarium fish toxicity: Of the six compounds, three (50 per cent) differ in rank by $1/6$ (1 position) or less of the total positions. Three (50 per cent) differ by $1/3$ (2 positions) or more. The P is 0.39, which is poor.

Accelerators are added to the rubber "mix" to hasten the molecular rearrangement of vulcanization. Various inorganic and organic substances have this effect but they differ greatly in potency. Organic sulfur compounds are especially potent. The Goodrich Company furnished three of the compounds

for the aquarium tests, but one was not sufficiently soluble to be toxic. Of the other two, 2-mercaptobenzothiazole (captax), which contains a benzene ring, is highly toxic (2 p.p.m.); 28 times more toxic than 4,5-dimethyl-2-mercaptothiazole.

SUMMARY

Hydroquinone when added to the aquarium water was found to be about a hundred times more toxic than phenol, to goldfish (and to *Daphnia magna*), but is only about twice as toxic when injected into fish or mammals. Tertiary butyl catechol shows a similar high toxicity in the aquarium, while the toxicity of catechol, resorcinol, and pyrogallol approaches more closely that of phenol.

As the substances of high aquarium toxicity are known to inhibit many oxidative and polymerizing autocatalytic "chain reactions," rank correlations were tabulated between the recorded inhibitory potency of various substances in these processes, and their aquarium toxicity for goldfish.

The correlation between aquarium fish toxicity and electric oxidation potential (P 0.09) is more than suggestive, and becomes still more so if explainable discrepancies are excluded. Antioxidant fat stabilizers show suggestive correlation with fish toxicity (0.20), and better with electric oxidation potential (0.10). The photographic reduction potential gives suggestive correlation with fish toxicity (0.20) and somewhat better with the oxidation potential (0.15). The gasoline induction period correlation is more than suggestive with the oxidation potential (0.099), but rather poor for fish toxicity (0.265). The rubber anti-aging potency gives only poor correlation (0.39) with fish toxicity. The reasons for these divergencies are not clear; they may perhaps be connected with the solvent properties of the substrate. As an example, Lea (p. 175) cites that 0.01 per cent of maleic acid prevents rancidity of fats, but is rendered ineffective by the presence of water.

Taken by themselves, no one of the P values is entirely convincing of the relationships stressed in this paper. However, the consistent finding of relatively small values of P lends considerable weight to the hypothesis that these chemicals act in a related manner; and that the chemical activity of a substance may furnish useful suggestions of its biologic potency, perhaps more so than the chemical constitution as such. The aquarium toxicity for goldfish is a convenient means of classifying the biologic potency.

CONCLUSION

There is considerable correlation between the aquarium fish toxicity and the antiautocatalytic potency of quinones, phenols, and related substances, in marked contrast to their toxicity on systemic administration.

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OXYGEN TENSION MEASUREMENT BY A METHOD OF TIME SELECTION USING THE STATIC PLATINUM ELECTRODE WITH ALTERNATING POTENTIAL

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There has been a well recognized need for an adequate method of measuring oxygen tension in small volumes during short time intervals without disturbing movement and without toxicity to living cells. Conventional polarographic technique provides the kind of data desired, but its dropping mercury electrode obviously fails to meet the requirements.

The static platinum electrode has been used in polarographic analysis (Laitinen and Kolthoff, 1942) but a number of difficulties are encountered: (1) the long time required for observation, (2) instability, and (3) lack of reproducibility. In biological application of this electrode, Davies and Brink (1942) reported a day-to-day variability of 30 per cent and Roseman, Goodwin, and McCulloch (1946) interpret only relative values for oxygen. The recessed electrode used by Davies and Brink improves the stability and reproducibility, but imposes a long time for restoration between successive observations.

The rotating platinum electrode employed by Laitinen and Kolthoff (1942), though improving stability, not only introduces disturbing movement but lacks reproducibility. In like manner the flow system of Giguère and Lauzier (1945) imposes similar restrictions.

In this investigation the possibility has been explored of overcoming the difficulties attendant on the use of the open, static platinum electrode through the employment of a suitable pattern of alternating potential with techniques arising from a special consideration of the time aspects involved. Because a good many other investigators have considered the possibility of an alternating potential, our observations over a wide range of frequency and pattern will be reported as well as the choice which has yielded the most satisfactory results.

Since the shortest possible time between successive measurements was desirable our observations began at 30 C.P.S.¹ and were extended ultimately to quite low frequency, 6 to 12 C.P.M.² Since these observations developed

¹ Cycles per second.

² Cycles per minute.

information essential to an understanding of the phenomena and the inherent limitations encountered and although they are preliminary in character they will be reported in the order of development. Initially a square wave potential pattern was selected for study, largely because of greater ease of interpretation. Subsequent findings support this as a desirable choice.

Oscilloscope Observations

Potential wave patterns were first produced by a photoelectric type wave generator in which two sector discs cut to produce the desired wave form were rotated between the light source and each of two photo tubes operating through a single amplifier. The pattern of potential thus produced was

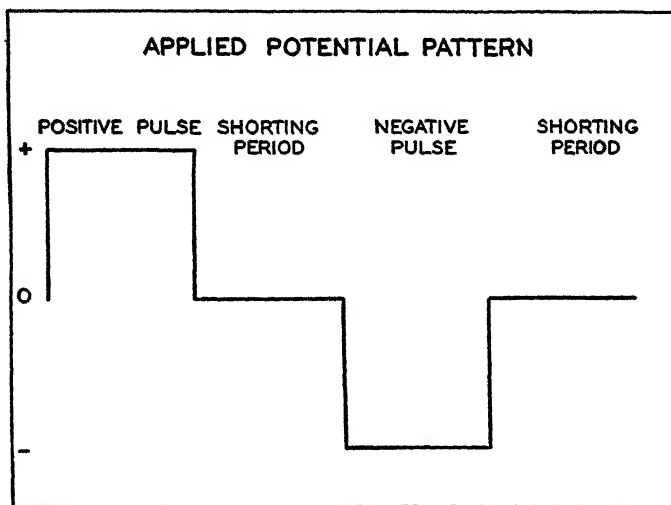


FIG. 1. See text.

imposed on an electrolytic cell composed of a platinum electrode of 20 gauge wire about 3 mm. long, *versus* a $M/10$ calomel half cell both immersed in a solution of $M/10$ KCl. An external resistance was introduced in series with the cell and the current pattern observed by following the potential difference across this resistance by means of a Dumont type 208B oscilloscope. A dual electrolytic cell was used as a means of comparing electrode performance in oxygen- and nitrogen-saturated KCl solutions. The square wave pattern chosen (Fig. 1) included a shorting period of no applied potential between each pulse, providing a "rest" interval during which the charge accumulated during the previous pulse is largely dissipated. Typical resulting current patterns for a frequency range of 5 to 30 c.p.s. at diffusion limiting potentials are shown in Fig. 2. These current patterns are repeated from cycle to cycle and appear stable on the oscilloscope screen.

It will be noted that greater influence of oxygen occurs at the end of each pulse and the beginning of each shorting period than elsewhere in the cycle, and that of the negative pulse slightly exceeds that of the positive. It can be seen from Fig. 3 that this influence of oxygen on the negative pulse terminal current increases with diminishing frequency in the range of 14 to 2.5 c.p.s. (pulse duration of 0.0178 to 0.1 second). This is not the case for the positive pulse terminal current as seen in Fig. 4. Fig. 3 shows that even with a pulse duration of 0.1 second there still exists a considerable (10 per cent) deflection for tank nitrogen which cannot be attributed to its O_2 content of about $1\frac{1}{2}$ per cent. In view of the trend, both considerations suggest continuing to still

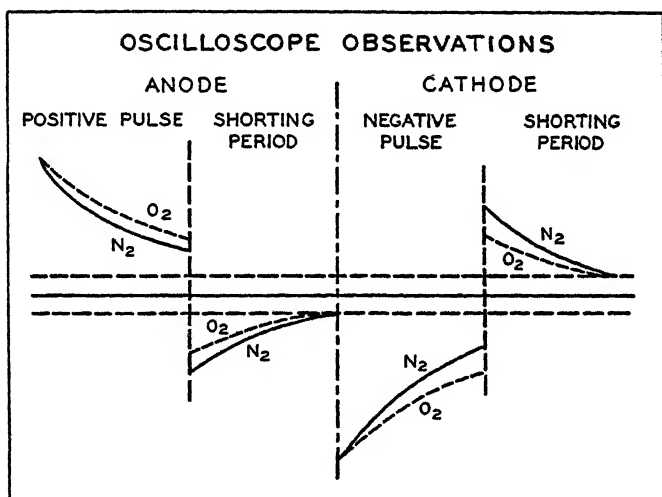


FIG. 2. Typical current patterns for oxygen- and nitrogen-saturated solutions when the potential pattern of Fig. 1 is applied in the frequency range of 5 to 30 c.p.s.

lower frequencies. This required a change in technique since the lower limit of flat frequency response of the oscilloscope used was about 2.5 c.p.s.

Oscillograph Galvanometer Observations

Subsequent observations at lower frequencies were made with Westinghouse "ultrasensitive" galvanometers adapted for continuous photographic recording. They provide a flat frequency response from 1500 c.p.s. to d.c. and a sensitivity of 100 μ a/inch when used with an optical lever of 15 inches. These are oil-damped galvanometers of the moving permanent magnet type with two field coils. In order to secure adequate deflection a single stage push-pull D.C. amplifier with an input resistance of about 25,000 ohms was introduced.

Since a more stable zero and more accurate potential pattern than those

obtained with the photoelectric type wave generator were desirable a cam-driven microswitch arrangement was substituted. This produced the same square wave potential pattern described previously.

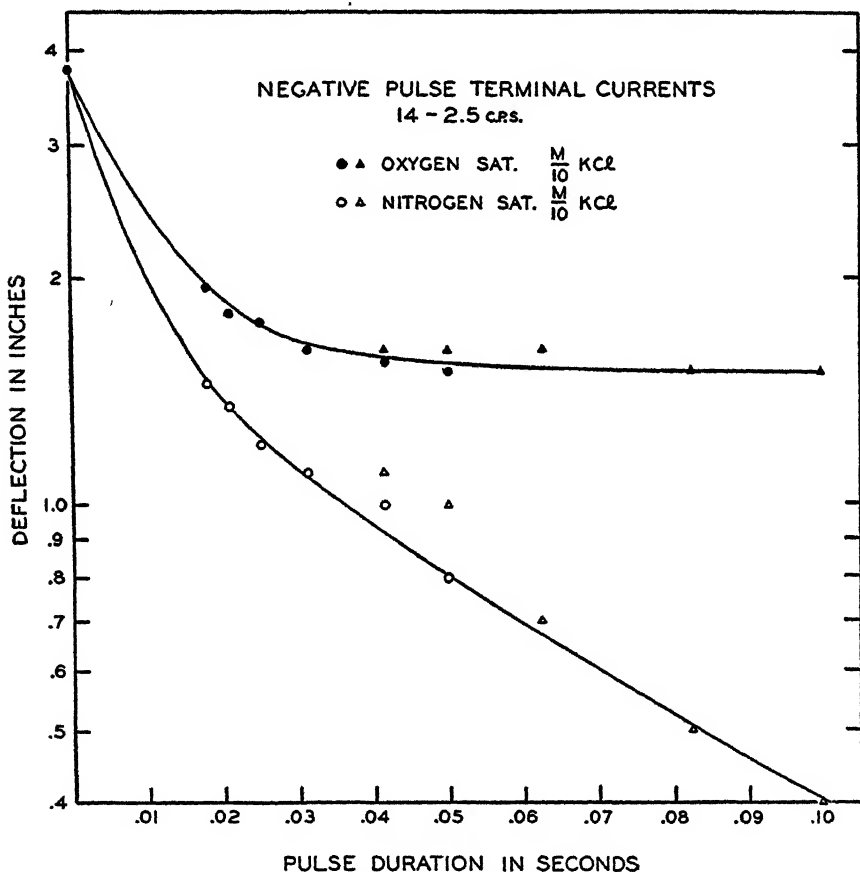


FIG. 3. Effect of pulse duration on negative pulse terminal currents due to high oxygen and low oxygen for frequencies in the range of 14 to 2.5 c.p.s. Current expressed in deflection recorded from oscilloscope screen.

Significance of Current-Time Patterns.—In Fig. 5 the current-time curve during a negative pulse of 0.7 second duration (about $\frac{1}{3}$ c.p.s.) is plotted for both oxygen- and nitrogen-saturated solutions. The long linear portion shown on this semilog plot when oxygen is very low, suggests the behavior of a condenser with a time constant of about 0.4 second. Thus a simple³ interpretation of our observations is that in the absence of oxygen we have

³ A more complete and rigorous consideration of the time course of current will be presented in a later article by one of us.

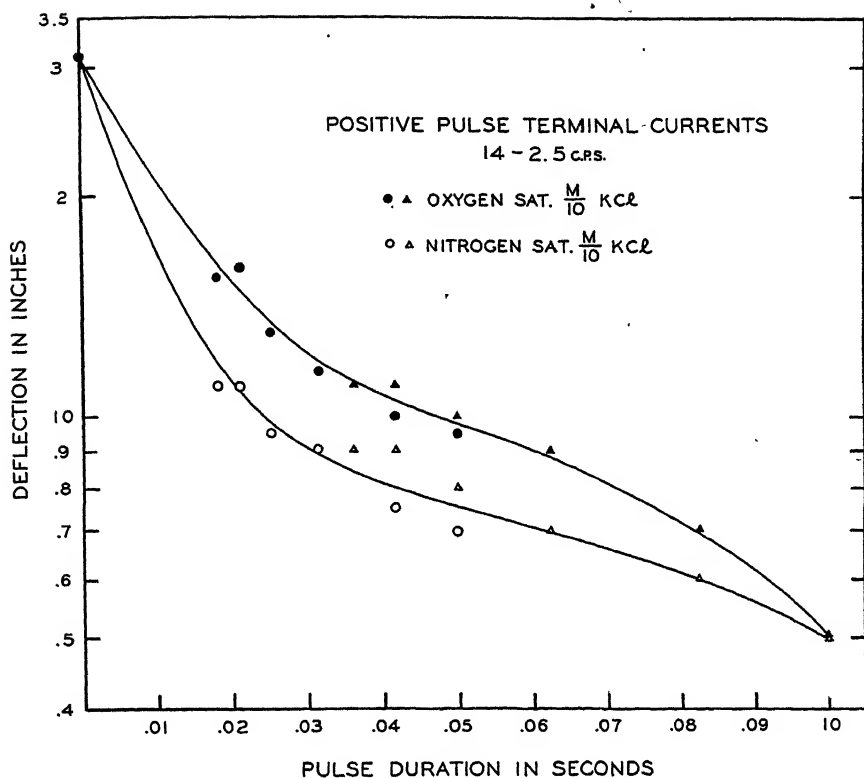


FIG. 4. Effects of pulse duration on positive pulse terminal currents for high and low oxygen.

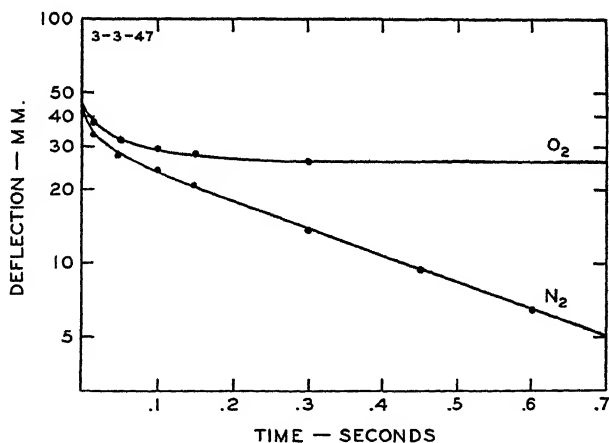


FIG. 5. Current-time curves during a negative pulse of 0.7 second duration (about $\frac{1}{3}$ c.p.s.) in oxygen-saturated and nitrogen-saturated $\frac{M}{10}$ KCl solutions. Current expressed in millimeters of deflection of oscillograph galvanometer.

during each positive and negative pulse the simple charging of a capacitative surface, followed during the shorting phase by the discharge of this surface. The presence of oxygen permits a conduction through the surface resulting in a sustained current during each pulse and a corresponding reduction in the discharge current during the subsequent shorting phase.

After a longer pulse duration the oxygen influence on the positive phase disappears, whereas it is enhanced in the negative phase. To avoid the background current in the absence of oxygen, if correctly interpreted as a capacitative charging current, three alternatives appear promising. (1) To reduce the time constant by reduction in external resistance; (2) to proceed to longer time intervals; (3) to cancel out the alternating component due to charge and discharge of the surface and measure only the rectified current component remaining chiefly from the negative pulse.

The last alternative was tested readily and resulted in observations which were satisfactory in magnitude of oxygen dependence and without background contribution in the absence of oxygen. The relation of response to oxygen concentration, however, proved to be non-linear of a character suggesting adsorption dependence; *i.e.*, very sensitive to low concentration but approaching saturation in the vicinity of 20 per cent.

A similar non-linear dependence was found for the oxygen response during the time range where there remained an appreciable background of capacitance charging current (Fig. 6).

Accordingly both remaining possibilities—reducing time constant and also lengthening the duration were explored. If the duration was made too great the instability common to the older static electrode techniques was encountered. However, reducing the time constant by cutting the external resistance below 2,000 ohms yielded satisfactory linearity of oxygen dependence for the terminal current of the cathode pulse. Typical current-time patterns recorded under these conditions are shown in Fig. 7.

Our interpretation is that with a short time constant, the capacitative current decays to a negligible value in time to observe the diffusion limited current before the diffusion wave extends far enough from the electrode to be subject to thermal convective interference and mechanical disturbances.

The initial oxygen influence is attributed to the discharge of an adsorbed layer of oxygen. Later the diffusion limitation asserts itself thus resulting in linear dependence. The discharge of the adsorbed oxygen appears to closely parallel the capacitative charging period.

This view was supported by the finding that during bubbling the deflection increased but became non-linear. Here we assume the diffusion limiting zone was swept away leaving again the adsorbed layer as the controlling factor (Fig. 8).

A family of current voltage curves for different durations are shown in

Fig. 9 with reduced external resistance. Here it will be seen that the typical diffusion limited plateau is developed for longer pulse duration and is absent for shorter durations. Thus the linearity of oxygen dependence parallels the development of the plateau. Refer to A, Fig. 7, and lower curve, Fig. 8.

These findings form the basis for the final instrument to be described.

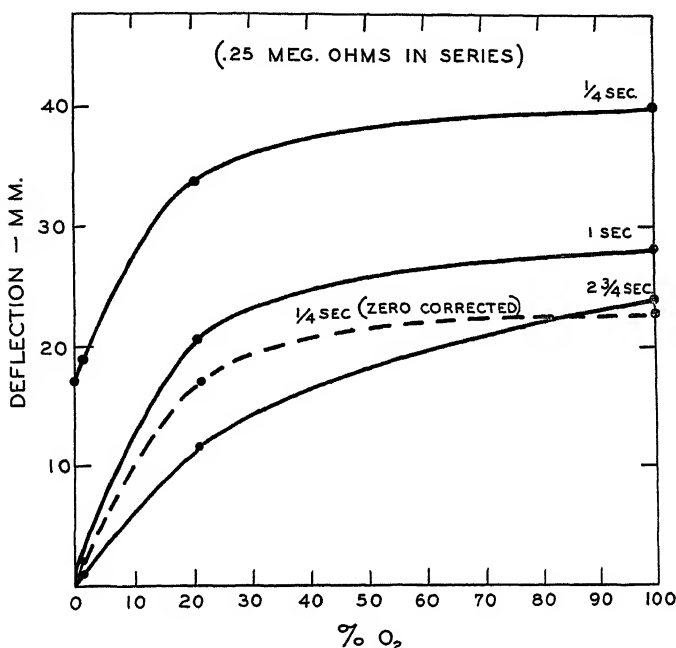


FIG. 6. Family of curves showing non-linear oxygen dependence of negative pulse terminal currents in the pulse duration range of appreciable background capacitive charging current. A resistance of 0.25 megohm was introduced into the external circuit during these observations.

Description of Present Apparatus

In the application of the electrode two types of recording are required (1) recording at increasing voltages in order to obtain current-voltage curves analogous to the typical polarogram for the identification of ions discharged, and (2) continuous recording at an appropriate voltage for diffusion limited discharge of the ion whose concentration is to be followed. Application of the preceding analysis of current-time curves requires that the current in the above cases be recorded at the end of each successive negative pulse. This is accomplished in our technique by a cam-microswitch arrangement which, in addition, produces the potential wave form. A stepping relay

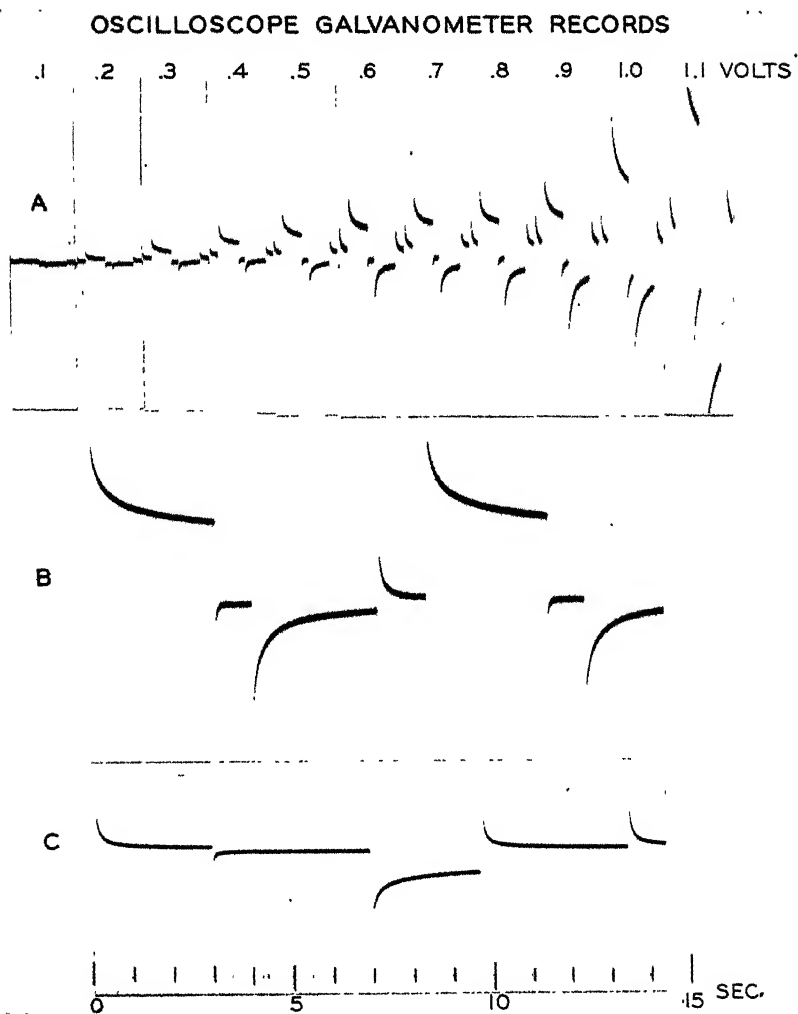


FIG. 7. Typical current-time records. *A*, composite of representative cycles for each 0.1 volt applied potential showing development of plateau. Upper pulse is negative. *B*, current-time curve in O_2 -saturated solution for potential pattern with diminished shorting period (10 sec./cycle). *C*, current-time curve in air-saturated solution (lower pulse is negative).

provides a means of automatically increasing amplitude in steps for the optional recording of current-voltage curves. The circuit diagram of this instrument is shown in Fig. 10. Microswitches (single pole double throw) MS_1 and MS_2 each connected to potential sources of opposing polarity are

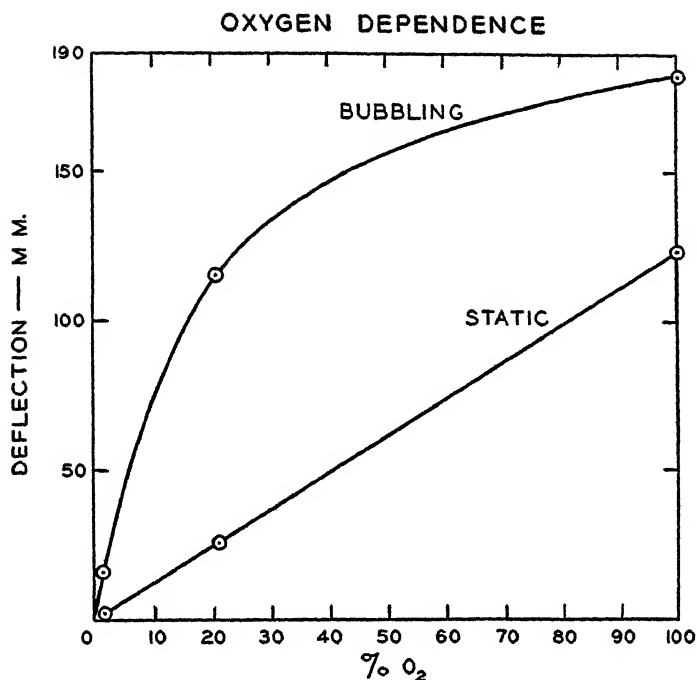


FIG. 8. Influence of stirring due to bubbling solution with gas mixtures.

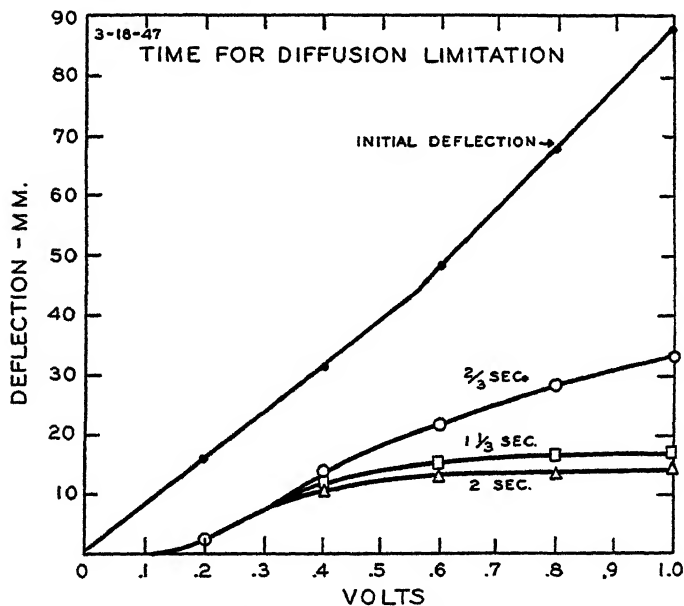


FIG. 9. Family of current-voltage curves for different durations during a negative pulse of 2 seconds in air-saturated solution showing development of the oxygen plateau with time.

placed at diametrically opposite switching positions with respect to the axis of a cam. The cam is of such a shape that each microswitch is closed during approximately a quarter of a revolution. As the cam rotates and closes MS_1 a negative pulse is imposed on the platinum electrode across the electrolytic cell. When the cam continues to revolve through the next quarter revolution and allows MS_1 to open, the cell is shorted through R_{15} , R_{13} , and R_2 to R_{12} depending on the position of the stepping switch. At the beginning of the third quarter revolution MS_2 is closed and a positive pulse is imposed on the cell. The final quarter revolution provides another shorting period and the whole sequence produces the wave form used in the preceding experiments.

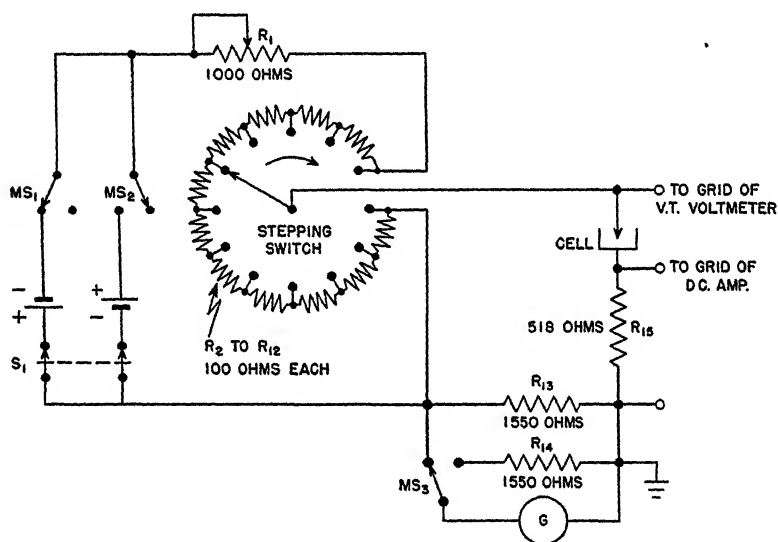


FIG. 10. Circuit diagram (see text).

In order to record only the negative pulse terminal current an additional cam is provided on the same shaft, which operates microswitch MS_3 (single pole double throw) just before MS_1 is opened. This introduces the current at the end of each negative pulse to galvanometer G , a Cambridge galvanometer of 0.83 second period, sensitivity 140 mm./ μ a (112 mm./ μ a with shunt) and critical damping resistance 1890 ohms, adapted for photographic recording.

The resistances R_{13} and R_{14} provide sufficient damping to allow for good registration of maximum deflection and zero values respectively. The record appears as a series of points each representing the maximum deflection or current flowing at the end of each successive negative pulse. R_{15} is provided as a load resistance for the D.C. amplifier associated with a Westinghouse oscillograph galvanometer in order that current-time patterns may be studied

under various conditions of electrode, supporting solution, etc. This, however, is used in connection with further development of techniques and is not essential for routine measurements.

In order to record current-voltage curves or polarograms during the course of experiment, an automatic stepping switch is combined with precision resistance R_2 to R_{12} as a voltage divider to increase stepwise the amplitude of the applied potential wave. This stepping switch is actuated by an additional cam and microswitch combination geared to the mechanism described above.

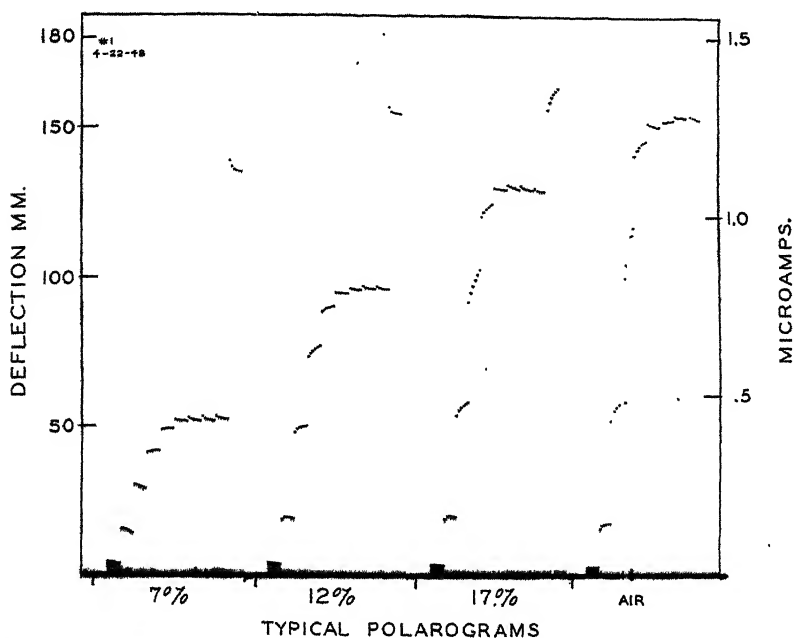


FIG. 11. Stepwise current-voltage records used in calibration. (Record for $1\frac{1}{2}$ per cent O_2 not shown.)

Since increasing the potential stepwise from zero requires several cycles (see Figs. 11 and 17) at each step before equilibrium is established the stepping cam is geared to the commutating cam in a ratio of the number of swings per step desired. At present six per step are being used and polarograms are plotted from the final swing of each step. This system avoids the manual counting of cycles and provides accurate timing of voltage increase with respect to the phase of applied potential wave. R_1 is provided in the circuit to allow for the total potential range and the degree of change per voltage step.

Electrodes.—Electrodes used at present are of $25\ \mu$ diameter platinum wire embedded in soft glass capillary drawn out to about 3 mm. This small di-

ameter affords a better metal-to-glass bond and provides better stability than the larger type. The length of exposed platinum wire is about 4 mm.

These electrodes are incorporated with a calomel half cell in an assembly designed for concentric entry into a conventional single opening absorption cuvette of 1 cm. path as shown in Fig. 12. The salt bridge is flushed by in-

RESPIROMETER ELECTRODE ASSEMBLY

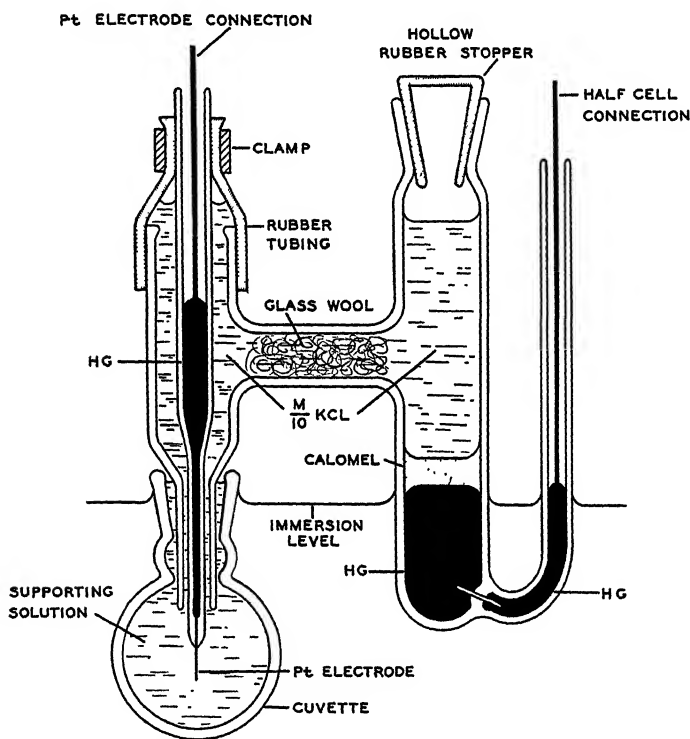


FIG. 12. Diagram of electrode assembly. Cuvette is about 15 mm. in diameter.

section of a hypodermic needle through the hollow rubber stopper. The assembly is clamped in a metal holder arranged so that the cell may be opened and closed with proper alignment of electrode and cuvette to avoid bending or breakage of the platinum when changing solutions or suspensions. The assembly is immersed in a constant temperature bath to the level indicated in the diagram.

Calibration.—Calibration of each electrode is accomplished by measuring deflection in solutions saturated at atmospheric pressure with known oxygen-

nitrogen mixtures in the range of oxygen percentage between air and pure nitrogen.⁴ These solutions may be saturated simultaneously and then introduced interchangeably into the cuvette by means of a hypodermic syringe taking care to avoid contamination by room air. The needle is introduced into the bottom of the cuvette and the solution slowly expelled until it slowly displaces the air and overflows by a volume several times that of the cuvette. Nitrogen-saturated solutions showed no measurable contamination by air when carefully manipulated in this manner.

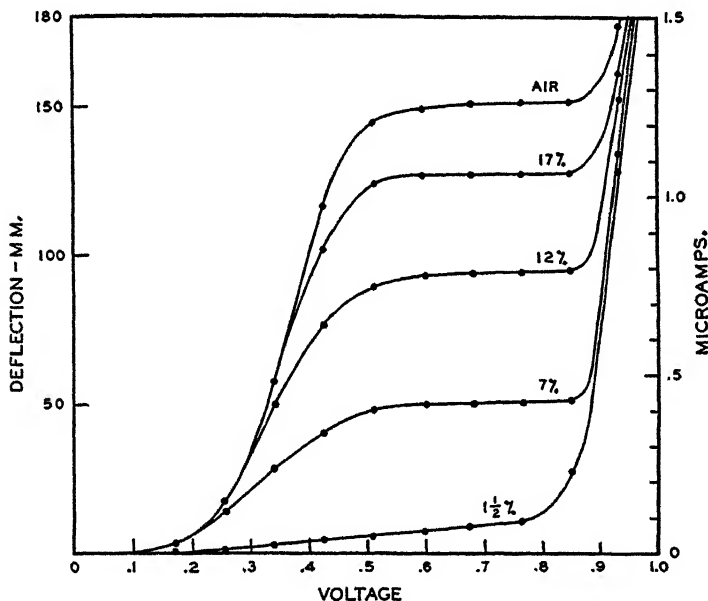


FIG. 13. Curves plotted from final point on each step of records shown in Fig. 11.

A typical set of current voltage records for $M/10$ KCl solutions saturated with tank N_2 ($1\frac{1}{2}$ per cent O_2), 7 per cent O_2 , 12 per cent O_2 , 17 per cent O_2 , and room air (20.9 per cent O_2) is shown in Fig. 11. Polarograms plotted from the final deflection value at each step are shown in Fig. 13. Attention is called to their similarity to ideal polarograms. The resulting calibration curve for this experiment is shown in Fig. 14 where the deflection or current at 0.7 volt for each solution is plotted against percentage saturation. The

⁴ Oxygen concentration where presented as per cent oxygen refers to saturation at atmospheric pressure.

While the method actually measures pO_2 , or oxygen tension, for purposes of comparison it is frequently more convenient to give the resulting current in a μa or galvanometer deflection.

linearity of the curve appears good, but it should be called to attention that galvanometer deflection can be read to less than 0.5 mm. (or 0.3 per cent), while the known percentage saturation of the calibrating solutions depends upon analysis of the gas mixtures and is accurate in this case only to the order of 1 per cent.

Stability.—Stability experiments with air-saturated M/10 KCl in the cuvette provided a point-to-point stability within a few tenths of a millimeter with the exception of an initial and diminishing decay of about 10 per cent extending over the first minute or two interval after the circuit is closed. Long period

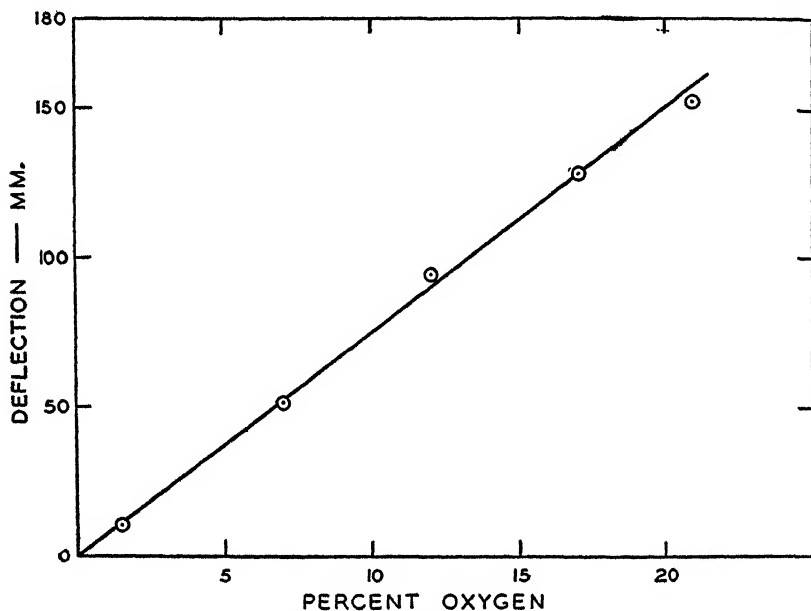


FIG. 14. Calibration curve.

stability experiments during several hours with the same electrode gave a day-to-day stability ± 1 per cent for air-saturated M/10 KCl. Typical examples are found in Fig. 17 and in the blank in Fig. 15. Not all electrodes provided such good results, however, and the variability in electrode behavior suggests some difference in the nature of the surface and its adsorbed gas film or the bonding between metal and glass. Electrodes often functioned perfectly for a period of months and then became useless because of irreproducible variation. These were easily replaced by spares which were always kept available.

Respiration Experiments with Yeast.—*Saccharomyces cerevisiae*, strain 10275 obtained from the American Type Collection, Washington, D. C., and cultured

on dextrose agar at 25°C. was removed from the agar and the cells washed twice by centrifuging. After transferring to filtered M/10 KCl + 2 per cent glucose buffered at pH 6.4 by M/10 phosphate buffer to make a suspension of the desired concentration, the suspension was then placed in the cuvette and air-saturated by bubbling prior to closing the cuvette. During this interval the platinum electrode was kept suspended in M/10 KCl to prevent drying out and the formation of salt crystals on the surface of the platinum. After closing the cuvette the rate of oxygen uptake was then recorded by

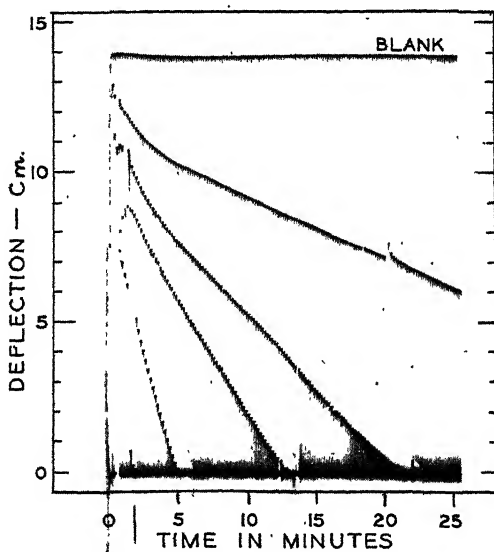


FIG. 15. Composite record of oxygen uptake by yeast suspensions in M/10 KCl buffered at 6.2 + 2 per cent glucose. Suspension of 2×10^8 cells per cc. diluted 1:0, 1:1, 1:2, 1:5.

the Cambridge galvanometer as described previously. Typical resulting curves are shown in Fig. 15.

Limitations of the Method.—In order to carry out valid measurements of oxygen uptake in a suspension of living cells the supporting medium or solution must provide the conditions of pH, total salt concentration, and composition within the range required to maintain normal metabolism by the material studied. The polarographic technique of analysis also makes certain requirements of the supporting medium in order to obtain conditions for diffusion-limited ion discharge. Its application to analysis of the medium surrounding living cells is therefore limited to the range where the biological and the electrochemical demands overlap. This range, of course, will vary with the requirements of the particular biological material investigated.

In the case of yeast respiration, for example, a pH range from 4.5–7.0 is desirable for uninhibited growth and respiration. There is, however, a definite lower limit to the pH which provides reliable polarographic data with platinum electrodes. In the typical polarogram with these electrodes current increases with increasing voltage until discharge is limited through ion concentration by diffusion to the electrode. The diffusion-limited current then levels off to a plateau until the potential for H^+ ion discharge is reached. The current at this point increases sharply in proportion to hydrogen discharge and masks that due to oxygen discharge. The hydrogen discharge potential is dependent upon the concentration of H ions and its intercept value at zero current is lowered with increasing concentration in the manner of a hydrogen pH electrode. The useful pH range of oxygen analysis is thereby limited to the range in which the oxygen diffusion-limited current is not masked by the hydrogen discharge current. In addition, the pH change due to CO_2

TABLE I
Current due to H^+ Ion Discharge

pH	0.51 volt	0.59 volt	0.68 volt	0.76 volt	0.85 volt
8.20	0	0	0	0.1335	1.482
7.51	0	0	0.0089	0.978	+ Off scale
6.72	0	0	0.0534	Off scale	" "
5.78	0	0.01781	0.623	" "	" "
4.95	0.0089	0.0623	0.712	" "	" "

Current in microamps. for representative applied potentials at different values of pH. Measurements carried out in buffered solutions oxygen-depleted by yeast suspensions.

evolution, etc. during an experiment, must not be great enough to produce hydrogen discharge at the potential chosen for the measurement of oxygen concentration on the oxygen diffusion-limited current plateau. The supporting medium must therefore be buffered at a pH sufficiently alkaline to avoid the interference of hydrogen discharge current, and with sufficient buffer capacity to tolerate the change introduced by the total CO_2 production during an experiment.

Table I shows the results of a series of experiments carried out with yeast suspensions of about 2×10^8 cells/cc. at $16.2^\circ C$. in $m/10$ KCl buffered at pH 8.20, 7.51, 6.72, 5.78, 4.95 with phosphate buffer at the concentration of $m/10$. The suspensions were allowed to remove oxygen from the solution until the current deflection at 0.59 volt reached zero or approached a constant value. A C-V curve of each buffered, oxygen-depleted solution was then recorded. The effect of increasing hydrogen ion concentration on the shifting of the hydrogen discharge potential may result in an elevation or current contribution in the absence of oxygen.

The results indicate that under the above conditions of buffering the method can be applied to respiration studies of yeast cells down to about pH 5. Background current due to H^+ discharge can be avoided at this pH by measuring the oxygen diffusion-limited current at about 0.55 volt. Current measured at a potential lower than this approaches the shoulder or beginning of the diffusion-limited current plateau and makes for instability. In like manner other organisms with different pH tolerances, medium composition requirements, CO_2 output, etc. will impose different limitations on the application of the method.

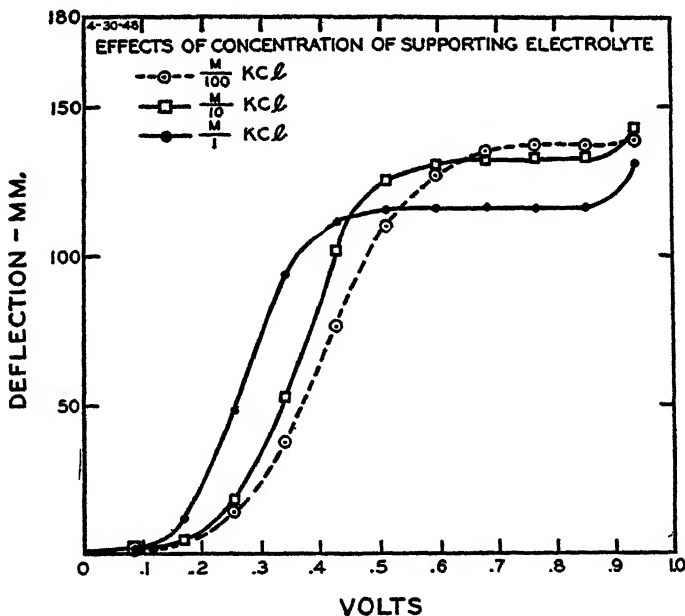


FIG. 16. See text.

The conventional polarographic technique of analysis requires a supporting electrolyte. The choice of this ion and its concentration depends upon the polarization characteristics of the ion deposited for analysis, and in the case of oxygen a solution of KCl serves effectively as a supporting electrolyte in the molar concentration range of one to one hundredth. In the case of yeast $M/10$ KCl is tolerated with little inhibition by osmotic effects. Such a solution was buffered at pH 6.4 by $M/10$ phosphate buffer and used with success in respiration experiments as shown in Fig. 15. In order to determine the feasibility of extending the application of the method to organisms with widely different osmotic requirements, polarograms were recorded for air-saturated solutions of $M/1$, $M/10$, and $M/100$ KCl. The results are shown in Fig. 16.

The diffusion-limited currents are in accord with predicted values for dissolved O_2 at these KCl concentrations, but the decomposition potential becomes more positive with increasing KCl. Concentrations within the entire range allow for usable polarograms, however, and the osmotic requirements of a wide variety of organisms should not limit the application of the method.

Time stability runs on blank air-saturated KCl solutions showed initial deflections decreasingly elevated above the final stable level of the curve. This initial decay prevents following oxygen changes during the first minute or two and attempts to correlate this decay with circuit and electrode characteristics were unsuccessful. Numerous variations in the ratios of pulse durations

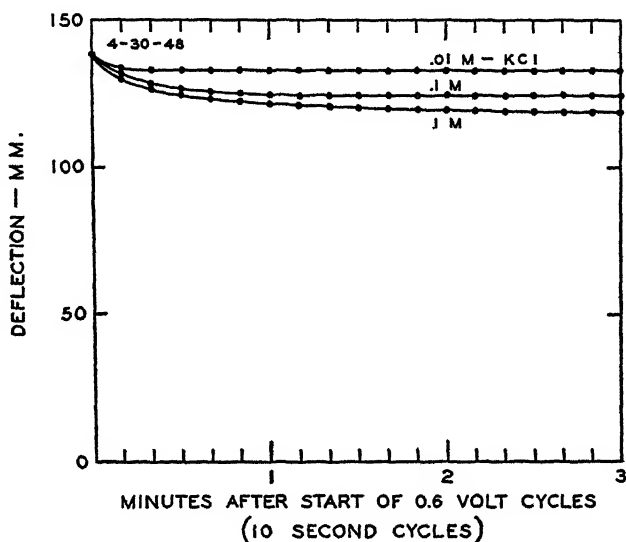


FIG. 17. See text. Effect of concentration on initial response.

to shorting periods were explored in this respect but without significant results. The concentration of the supporting solution has a marked influence, however, as shown in Fig. 17. Low KCl concentrations minimize the decay effect while high KCl concentrations enhance it. The decay is not minimized, however, in low KCl concentrations ($M/100$) when yeast is present. 2 per cent glucose solution and 0.5 per cent gelatin solutions also enhanced the decay suggesting a physical limitation of diffusion restoration worthy of further study.

The initial decay phenomenon further suggests that a similar lag may occur throughout the observations thus limiting the time resolution of the method. Results of rapidly changing the oxygen tension without disturbing the electrode or diffusion layer are shown in Fig. 18, curve *B*. This was accomplished by

illuminating a suspension of green algae in the cuvette for sharply defined periods. The sharp changes between the rise in oxygen tension produced by photosynthesis during illumination and the fall produced by respiration in the dark indicate that the effect of decay on time resolution is not evident.

However, a secondary photo-effect was observed in this experiment. This is shown by the effect obtained (curve *B*, Fig. 18) when the suspension of algae, was replaced by a blank solution of air-saturated KCl. A cusp of about 10 per cent increase in total current appeared during radiation. The effect was absent in oxygen-depleted solutions and appeared to be dependent

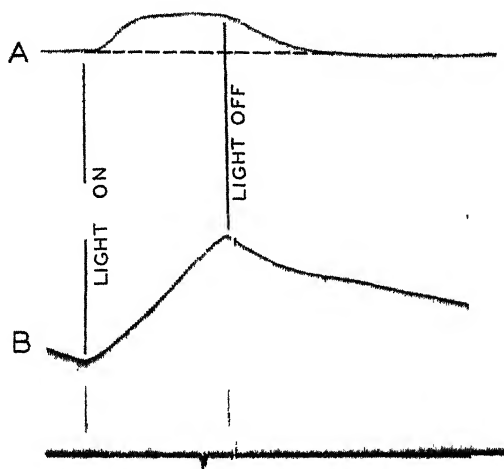


FIG. 18. Effect of visible radiation. *A*, in the absence of organisms. *B*, on suspended green algae showing time resolution and superimposed photo-action on electrode response.

on the amount of oxygen present. It persisted when solutions containing oxygen were radiated through a "heat-transmitting" filter but was effectively eliminated by introduction of an efficient "heat-absorbing" filter such as a 15 mm. thickness of $M/10$ cupric sulfate solution.

Influence of Metallic Ions.—Since many metal ions such as Cu^+ , Pb^+ , Sn^+ etc. are deposited in the potential range of the oxygen diffusion-limited discharge in conventional polarography it becomes desirable to determine effects of these ions under the conditions of the present method. Pb^{++} was chosen for study because of the clear vertical rise at the decomposition potential and the flat plateau in the range of the oxygen diffusion-limited current of its polarogram. $M/10$ KCl solutions were made up with Pb^{++} at 1×10^{-3} molar concentration and diluted with $M/10$ KCl to 7.5×10^{-4} , 5.0×10^{-4} ,

2.5×10^{-4} , and 1.0×10^{-4} molar. Recorded polarograms with the apparatus for these solutions showed an almost vertical rise at 0.65 volt with an extremely flat plateau from 0.68 to 0.85 volt. Good linearity is obtained by plotting the current measured at 0.76 volt against concentration and a day-to-day stability of ± 1 per cent was attained for Pb^{++} at $m/1000$ concentration. It is evident then that the method is sensitive to metallic ions in the same manner as oxygen and care must be taken to exclude from the supporting electrolyte those ions which are reducible in the potential range of the oxygen diffusion-limited current.

DISCUSSION

The application of a square wave potential to an electrode system is by no means a new idea, having been used for standing wave analysis of polarization and overvoltage phenomena by many investigators. Little use, however, has been made of its application to the current-time relations of diffusion-limited ionic discharge at fixed solid electrodes. In the present investigation current-time curves were studied under various conditions with the view of selecting a portion of the curve for use in oxygen analysis. The shorting period between positive and negative pulses was introduced to minimize the carry-over of current phenomena from one pulse to another. In other words the shorting period provides a "rest" interval during which charge developed by one pulse is largely dissipated before the current is developed by the following pulse of opposite polarity. This shorting period was chosen to be at least of sufficient duration to allow for dissipation of this charge to a low value. Without it current-time curves were difficult to interpret and utilize.

The exact nature of the process by which the applied potential wave form results in restoration of the oxygen diffusion gradient developed during each negative pulse is difficult to interpret from the data presented here. The time lags in reaching a stable value exhibited upon changing voltage stepwise and in closing the circuit at the beginning of an experiment both suggest that restoration is not complete with each cycle. Apparently some sort of an equilibrium is reached after a requisite number of cycles. The stability achieved in a relatively short period suggests that the diffusion layer developed is only a fraction of the thickness necessary to obtain a "steady state" in the conventionally used static platinum electrode. The present method, of course, depends upon accurate timing of commutation and measuring interval of the selected portion of each negative pulse.

The method has the advantage of utilizing simple stationary platinum electrodes with as good or improved stability as compared to those methods requiring moving electrodes, constant flow, or recessed electrodes. In addition, the mechanical and electrical system required to impose the wave

form and measure the diffusion current is not costly or difficult to assemble in the laboratory.

As a microrespirometer for cell suspensions the instrument has the advantages of high sensitivity, reproducibility, and rapidity of measurement. Since it is direct reading and recording it should find application where transient changes in respiration rate of several seconds' duration are experienced. Unlike the dropping electrode it is free from the toxic effect of mercury ions. In addition its use with conventional cuvettes as respirometer vessels provides facility for simultaneous measurements of radiation or spectral absorption during respiration or photosynthesis.

It has, of course, some limitations, several of which have been discussed. The initial decay experienced during the first minute or two of a respiration experiment does not impose a hardship except in those cases where the heaviest cell suspensions are used and the O_2 uptake rate is rapid enough to have appreciably depleted the solution during this period. In all other cases it is in fact desirable to delay recording for a few minutes, in order to establish temperature equilibrium after filling the cuvette or saturating with air, etc.

During continuous oxygen uptake measurements over long periods with large or heavy cells or aggregates which settle out of suspension due consideration must be given to means of periodic stirring since measurements cannot be obtained during actual stirring.

To avoid the photo-artifact previously described precautions must be taken to filter out the intense infrared common to incandescent lamps. In preliminary studies on the effects of ultraviolet radiation on respiration no difficulty was experienced at the physiological intensities used.

The limitations imposed by hydrogen ion concentration and salt concentration are similar to those encountered with conventional platinum electrodes, and for yeast respiration have been determined and discussed above. By determinations of both the biological and the electrochemical requirements the useful ranges for each organism can be determined without too much difficulty.

As a further application, the method should lend itself to *in situ* determinations of dissolved oxygen in tissues and body fluids. In addition the absence of physical stirring should provide ideal conditions for the study of the permeability and diffusion of oxygen through membranes and tissues.

The method furthermore lends itself to scaling down to micro-electrode dimensions for micrurgical application. With present electrodes of 25μ diameter and 4 mm. length (area of about 0.32 mm.^2) current deflection at air saturation equals about $1\mu\text{a.}$ This is suitable for a fast acting galvanometer but with micro-electrodes an amplifier will be required such as has been used in our oscillograph galvanometer current-time studies. With scaling down of electrodes increasing amplification is required because of diminishing current.

Correspondingly increased input resistance makes this readily possible. Since polar capacitance decreases with reduction in area as the internal resistance increases correspondingly, it is possible to provide this condition and still maintain the same RC constant with smaller electrodes. No difficulty in this direction is evident from present experiments and further work is projected along these lines.

In addition to application in biology the method should be of advantage in polarographic procedure in which mercury becomes undesirable such as unattended control measurements in industry or in the laboratory where anodic oxidation is to be studied.

SUMMARY

1. The possibility of obtaining sustained and reproducible results in the analysis of dissolved oxygen with simple platinum electrodes by means of the application of a periodic potential pattern was explored over a wide range of frequencies and with a variety of wave forms.

2. Satisfactory results were obtained by the application in the frequency range of 5 to 10 C.P.M. of a square wave consisting of a positive and a negative pulse with interposed shorting periods and observing the current flowing at the end of each successive negative pulse. This was found to be linearly proportional to O_2 concentration for a pulse duration of the order of 1 second when the RC constant of the circuit was sufficiently small.

3. An instrument was developed to provide the required wave form and record the terminal currents of the negative pulses. The instrument provides either for recording of current voltage curves (polarograms) or for continuous recording at a fixed voltage of diffusion limited current values.

4. Typical measurements of oxygen uptake with yeast suspensions illustrate the application of the technique to problems requiring frequent determinations during short intervals.

5. Applications of this technique to biological and other problems are indicated with its limitations.

Addendum.—In using solutions in the cell which differ markedly in molar concentration from the $M/10$ KCl solution in the salt bridge arm, it is desirable to introduce an agar plug made up with KCl . It is also desirable to eliminate pressure effects which may disturb the plug and force the bridge solution by the plug and into the cell.

In a recent application the bridge is connected and introduced by means of an agar plug at the bottom of a small tube with the electrode closing the top. This allows for a cell only 2 mm. in diameter and about 6 mm. long.

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CRYSTALLINE PNEUMOCOCCUS ANTIBODY

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The preparation of crystalline diphtheria antitoxin was reported in this *Journal* in 1942 (Northrop, 1942). The method of purification consisted in digesting the toxin-antitoxin precipitate with trypsin, which destroyed the toxin and liberated the antibody. The antibody itself was apparently also split during the reaction so that the product obtained was a derivative of the naturally occurring antibody. The present work was undertaken to see whether this method of purification was of general application. Type I antipneumococcus horse serum was used as the starting material and the writers are indebted to Dr. Jules Freund of the New York Department of Health for supplying them with large quantities of this serum.

In preliminary experiments the antibody was precipitated by the addition of Type I specific polysaccharide and the resulting precipitate treated with trypsin or pepsin at various hydrogen ion concentrations. The antibody complex in this case, however, is not hydrolyzed, as in the case of diphtheria antitoxin and no antibody could be recovered by this process. It appears, therefore, that the action of pepsin and trypsin on antigen-antibody complexes is determined by the chemical nature of the antigen in spite of the fact that hydrolysis occurs somewhere in the antibody molecule. This result is indicative of a primary valence union between antigen and antibody, as otherwise the chemical nature of the antigen would hardly be expected to influence the hydrolysis of the antibody.

Various other methods have been described for preparing pure (completely precipitable) antibody. Landsteiner, Gay and Chickering, Felton, Kirk and Sumner, Heidelberger and Kendall, and others (*cf.* Landsteiner, 1945) obtained preparations which were pure or nearly pure antibody, in the sense that they were completely precipitated by antigen, by dissociating the antigen-antibody complex in various ways. Chow and Goebel (1935) obtained similar preparations by precipitation of a concentrated antibody solution with acid potassium phthalate solution.

Preliminary experiments indicated that the latter method was the most efficient for the preparation of large amounts of pure antibody and a slight modification of this method was used in the present work. The method of preparation finally worked out consists essentially of the following steps. High titer antipneumococcus horse serum is diluted with water and the precipi-

tate, which contains nearly all the antibody (Felton, 1928), dissolved in normal saline. This solution is mixed with 0.2 M pH 3.6 acid potassium phthalate in certain proportions, which vary slightly with different lots of serum. This treatment precipitates the inert globulins and leaves the antibody in solution. The antibody obtained in this way is 90 to 100 per cent precipitable with the specific polysaccharide. It gives a slightly milky solution in neutral salts and is nearly homogeneous in the Tiselius apparatus with a migration velocity of 1.2×10^{-5} cm.²/volt sec. which corresponds to that of globulin from normal horse serum. (The writers are indebted to Dr. Gertrude Perlmann for carrying out these determinations.)

This antibody solution may be further fractionated by precipitation with ammonium sulfate into three main fractions, one of which is insoluble in neutral salts from pH 4 to pH 10, one which is soluble in neutral salts but is precipitated by 0.20 saturated ammonium sulfate. The remaining fraction precipitates between 0.20 and 0.35 saturated ammonium sulfate. It gives a clear bluish solution in neutral salts and contains most of the inert protein which survives the treatment with acid potassium phthalate.

The fraction precipitating at 0.2 saturated ammonium sulfate can be further separated into a fraction precipitating at 0.17 saturated ammonium sulfate and a small amount of antibody precipitating between 0.17 and 0.20 saturated ammonium sulfate. This last fraction may be crystallized by slowly stirring a saturated solution at 25° (in about 0.2 saturated ammonium sulfate) (Fig. 1). The crystals appear as rosettes, sometimes mixed with rods (Fig. 2). The faces are somewhat rounded in most cases and no really satisfactory preparation was obtained. Similar results have been observed in this laboratory with other proteins and have been due in most cases to the presence of more than one protein, or to the fact that conditions used for crystallizations are not quite correct. A large number of variations in conditions and precipitating agents were tried with no improvement and it is probable that the difficulty arises from the fact that the protein is unstable under the conditions required for crystallization and is partly changed to a less soluble form. This is indicated by the fact that the first crystals to appear are usually the best. Also, the insoluble protein formed during the first crystallization must be removed before recrystallization, otherwise the second crystallization yields much poorer crystals than the first. If the solution is not stirred, crystallization may start but soon stops owing to the fact that the crystals settle rapidly to the bottom of the beaker. The crystallization of pepsin is very similar since in this case also crystallization is much better and faster if the solution is stirred.

The largest part of the antibody finally collects in the fraction insoluble in neutral salts but soluble in dilute acid and alkali. The results indicate that some of this fraction is present in the original material while more is formed during the fractionation. If alkaline solutions of this insoluble protein are

mixed with solutions of the more soluble fraction, and then neutralized, the protein does not precipitate but the solution becomes cloudy. The original material used in these experiments had been stored at 5°C. for from 4 to 7 years in the presence of 0.5 per cent ether, 0.3 per cent phenol, and 0.005 per cent merthiolate. A precipitate had formed and settled out during this time and this precipitate resembles closely the insoluble protein isolated during the fractionation.

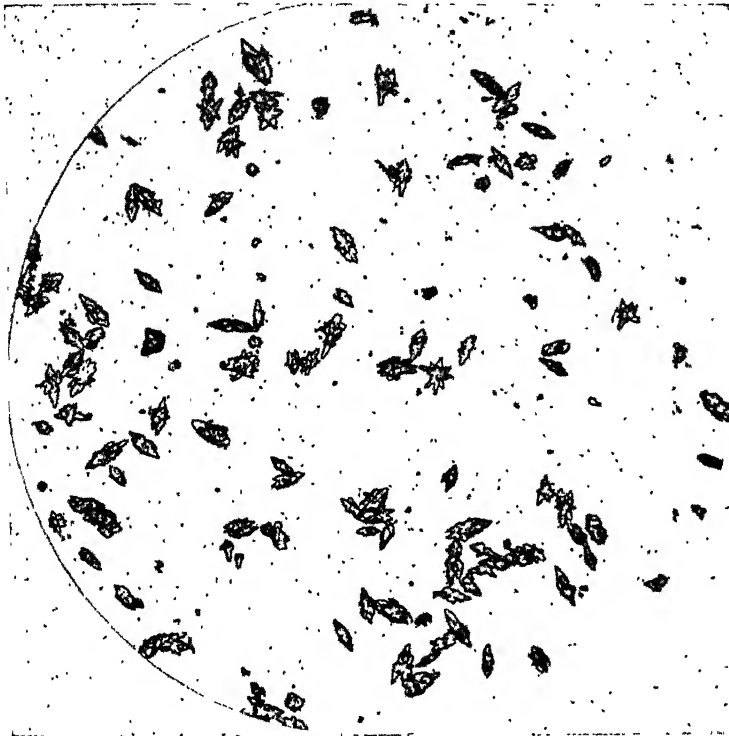


FIG. 1. Crystals of Type I pneumococcus antibody, preparation 152-51. $\times 130$.

The most soluble fraction gives a clear, bluish solution in neutral salts and yields highly refractile rounded particles but no definite crystals. This fraction is usually 80 to 90 per cent precipitable and no method was found to remove the inert protein.

None of these fractions is even approximately homogeneous by the solubility test (Fig. 3) and crystallization does not improve the purity as determined either by solubility or by precipitation with antigen. In fact, the crystalline material is, if anything, less pure since the protein is somewhat unstable under the conditions required for crystallization so that the formation of the crystal

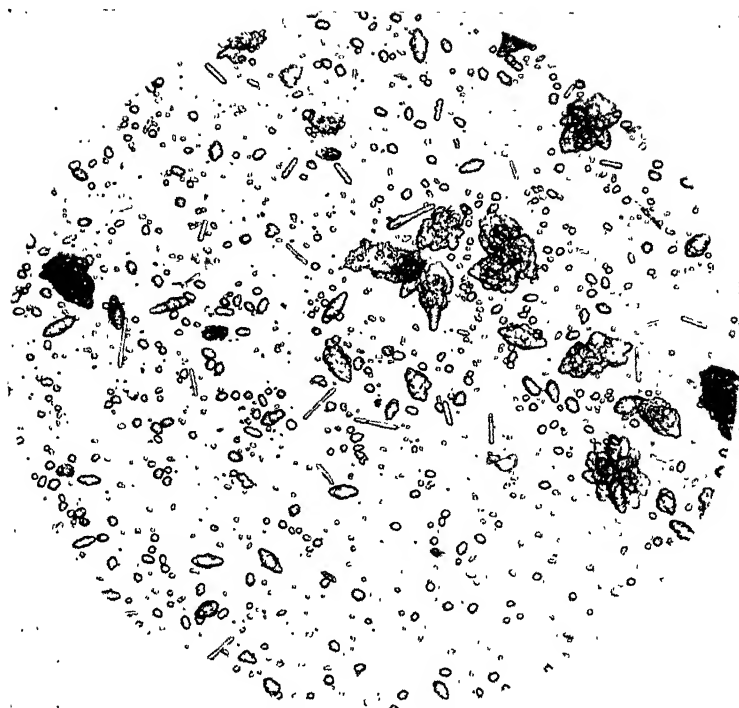


FIG. 2. Rosettes and rods. No. 72-201. $\times 65$.

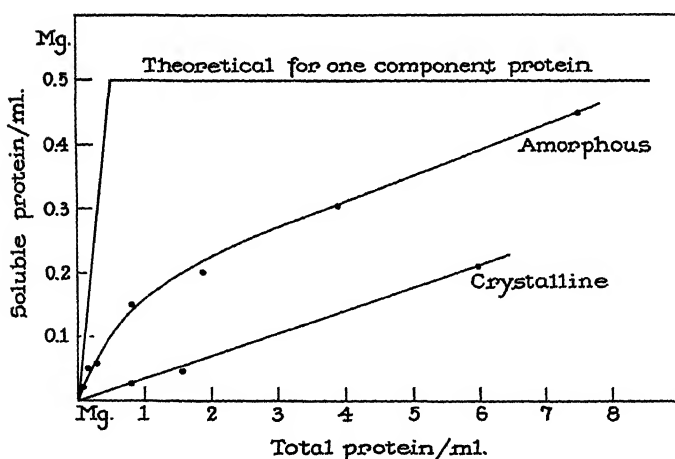


FIG. 3. Solubility of preparation 180-69 in amorphous and crystalline form in 0.33 saturated ammonium sulfate-0.17 M sodium acetate at 25°C. Two 5 ml. samples of suspension 180-69 were centrifuged. The precipitate in one tube was dissolved in 5 ml. 0.25 M sodium acetate (milky solution) and precipitated by the addition of 3 ml. saturated ammonium sulfate. This is the amorphous sample. Both crystalline and amorphous precipitates were washed four times with 2 ml. of the solvent and solubility determinations made on varying quantities of the precipitate as described (Northrop, Kunitz, and Herriott, 1948).

is accompanied by the formation of some less soluble protein. The same phenomenon was encountered in the crystallization of diphtheria antitoxin (Northrop, 1942).

A purified antibody preparation was also obtained by dissociating the immune precipitate. Immunological tests of these preparations showed that they differed quite markedly in their protective and agglutinating properties. They all reacted with rabbit antihorse serum.

EXPERIMENTAL

Methods

1. *Protein Determination*¹.—One ml. of solution containing 0.01 to 0.5 mg. protein per ml. is added to 9 ml. 0.3 M trichloroacetic acid. The tubes are placed in a boiling water bath for 1 minute and allowed to stand 1 hour or more at 25°. The turbidity of the samples is determined in a Duboscq type Klett photoelectric colorimeter, using 0.04 M CuSO₄ in 0.04 M H₂SO₄ as standard. The amount of protein is determined by comparing the observed reading with a standard curve prepared from determinations made on a series of samples of known protein concentration. The suspensions do not obey Beer's law and hence the values for the concentration cannot be calculated from the colorimeter reading in the usual way but must be read off from an empirical curve as described above. The slope of the curve varies with different proteins so that it is necessary to carry out the determinations with two different concentrations of protein.

If the two determinations agree, the result is probably correct within 5 per cent. If they do not agree, the protein is different from that used to prepare the standard curve and a new standard curve must be prepared. In the present experiments, the important figure is that for protein not precipitated by the polysaccharide. In most cases this is less than 10 per cent of the total protein, so that a 10 per cent error in the determination of non-precipitable protein represents an error of 1 per cent or less in the values for the precipitable protein (antibody-protein). If the precipitable protein is less than half the total protein, however, the error is much larger and the method cannot be used for protein solutions containing low proportions of antibody.

2. *Determination of Antibody Content*.—The solution is diluted with 0.1 M pH 7.6 phosphate buffer to contain 0.3 to 0.8 mg. protein per ml. Two ml. of the diluted solution is placed in each of four small test tubes and 1 drop of specific polysaccharide solution containing 0.5, 1.0, or 2.0 mg. polysaccharide per ml. added to each of three tubes. All four tubes are placed at 5°C. for 24 hours, centrifuged, and the protein determined in the supernatant. The lowest value found in the tubes containing polysaccharide is taken as the figure for the inert protein and the difference between this and the protein content of the control tube is the antibody-protein. Ammonium sulfate interferes with this determination if present in concentrations greater than 0.01 saturated.

3. *Precipitation with Acid Potassium Phthalate*.—The results obtained with this method depend on the concentration of protein, the pH and concentration of the acid

¹ The analytical work reported in this paper, and part of the experimental procedure, were carried out by Miss Marie King.

potassium phthalate, the temperature, and the length of time the solution is allowed to stand (*cf.* Chow and Goebel, 1935). Preliminary experiments indicated that the highest yields of completely precipitable antibody were obtained by adding 0.2 M pH 3.6 acid potassium phthalate to globulin solutions containing 25 to 30 mg. protein per ml. and allowing the solutions to stand 24 hours at 20°C. The exact amount of phthalate required varies with different lots of serum. In some cases the yield of antibody is the same over quite a wide range of potassium acid phthalate, but in some cases the correct range is quite narrow. In order to determine the optimum quantity of acid potassium phthalate to add to the antibody solution, 10 ml. of solution containing 25 to 30 mg. protein per ml. is pipetted into a series of tubes and varying quantities of pH 3.6, 0.2 M acid potassium phthalate added slowly with constant stirring. A precipitate forms at once and increases slowly for several hours. After 24 hours at 20°C. the suspensions are centrifuged and the supernatants analyzed. The results of two such preliminary tests are shown in Table I. Sample 180-6 gave

TABLE I
Purification of Crude Antibody Solutions by the Addition of Acid Potassium Phthalate

Amount of 0.2 M pH 3.6 acid potassium phthalate added to 10 ml. of solution	Per cent of total protein precipitated by polysaccharide		Antibody recovered	
	Sample 180-6	Sample 170-3	Sample 180-6	Sample 170-3
<i>ml.</i>			<i>mg.</i>	<i>mg.</i>
0	60	60	200	200
3	90	98	200	200
3.5		98		200
4	96	100	200	140
5	98		180	
6	100		170	
7	100		120	

pure antibody with the addition of 6 or 7 ml. phthalate whereas Sample 170-3 gave pure antibody with 4.0 ml.

The mechanism of the reaction is obscure. It does not appear to be either a salting out or a denaturation. The quantity of phthalate required is nearly proportional to the amount of serum. The precipitate formed does not digest with trypsin as rapidly as does a boiled suspension of the same protein. The protein, therefore, is probably not denatured. It seems probable that the precipitate is an insoluble phthalate salt of the inert globulins. In cases where the first precipitation fails to remove all the inert protein, no further purification is obtained by repeating the phthalate treatment.

Conditions for Crystallization

The amorphous protein precipitate is stirred slowly with about three times its volume of 0.05 saturated ammonium sulfate at 5°C. for 24 hours. The protein should dissolve completely to form a clear, bluish solution. If there is more than a slight turbidity, the suspension should be filtered by gravity through No. 3 Whatman

paper, or centrifuged at high speed. The protein is then precipitated in the amorphous form by bringing the solution to 0.25 saturated ammonium sulfate, the precipitate centrifuged out and dissolved in 0.05 saturated ammonium sulfate, as described above.

If the precipitate forms a clear or nearly clear solution, the protein content is determined and the solution diluted to give a protein concentration of about 15 mg./ml. The pH is adjusted to about 6.5 and the solution stirred slowly. The stirring rod should be near the bottom of the beaker and should not cause any foam to form. Saturated ammonium sulfate is added very slowly from a dropping funnel until the

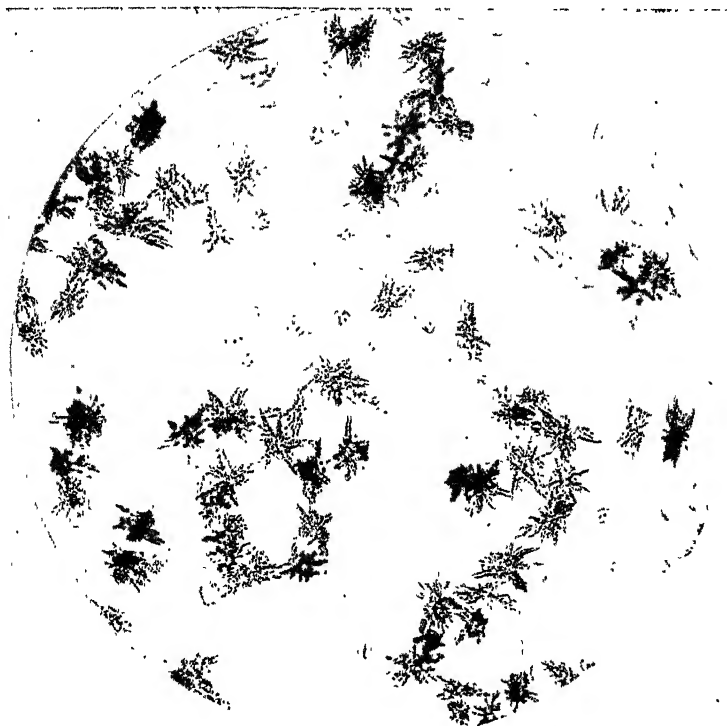


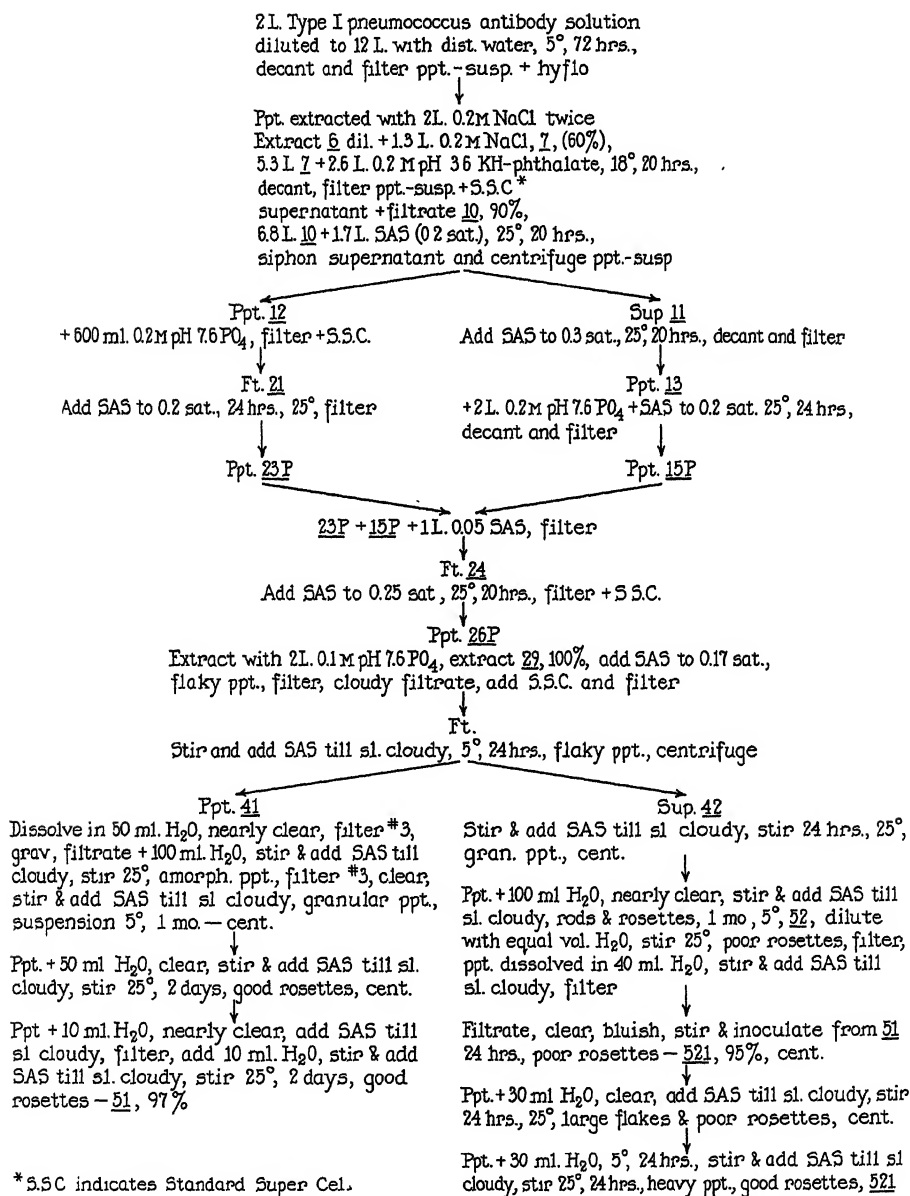
FIG. 4. Antibody "flakes," preparation 171-13, June 1, 1948.

solution is slightly cloudy. The stirring is continued and the precipitate should become rapidly heavier.

The precipitate is examined under the microscope and should consist of small irregular rosettes. The points look sharp under a low power (50 to 70 diameters) but under higher magnification appear rounded. Some preparations yield large, regular flakes, which look very much like snowflakes (Fig. 4). They appear to be made up of very small refractile granules arranged in a more or less regular pattern. These "flakes" are insoluble in 0.05 saturated ammonium sulfate and should be filtered off, if they form. If the precipitate is amorphous, it should be filtered off and the filtrate stirred at about 25°C., without the addition of more saturated ammonium

Diagram 1

Preparation of Crystalline Fractions 152-51 and 152-521



* S.S.C. indicates Standard Super Cel.

TABLE II
Experiment 152

	No.	Vol.	Pro- tein/ml.	Anti- body	Total AB
		ml.	mg.	per cent	gm.
6 liters of Type I antipneumococcus horse serum diluted to 60 liters with distilled water, 5°C., 48 hrs. Supernatant siphoned off and precipitate centrifuged. Precipitate dissolved in 2 liters 0.1 M NaCl. 0.5 per cent ether, 0.3 per cent phenol, and 0.005 per cent merthiolate added and the solution allowed to stand at 5°C. for 6 yrs.....	1	2000			
2 liters <i>No. 1</i> diluted to 12 liters with water, allowed to stand 72 hrs. at 5°C., supernatant decanted, precipitate-suspension (4 liters) + 200 gm. Hyflo, filtered by suction on 235 cm. E and D No. 303 papers, precipitate + 2 liters 0.2 M NaCl, filtered.....	4	2000			
Precipitate + 2 liters 0.2 M NaCl, filtered.....	5	2000			
<i>No. 4 + 5</i>	6	4000	36	60	80
4 liters <i>No. 6</i> + 1.3 liters 0.2 M NaCl.....	7	5300	27	60	80
5.3 liters <i>No. 7</i> + 2.6 liters 0.2 M pH 3.6 potassium acid phthalate, 18°C., 20 hrs., supernatant siphoned off, supernatant + 200 gm. standard Super Cel, filtered on 35 cm. No. 303 E and D paper.... Filtrate.....	8				
Precipitate-suspension + 100 gm. Hyflo + 100 gm. standard Super Cel, filtered..... Filtrate.....	9				
<i>No. 8 + 9</i>	10	6800	9	90	55
6.8 liters <i>No. 10</i> + 1.7 liters saturated ammonium sulfate (0.2 saturated) 25°C., 20 hrs., supernatant siphoned off..... Supernatant..	11				
Precipitate-suspension..	12				
6 liters <i>No. 11</i> + 2 liters saturated ammonium sulfate (0.3 saturated), 25°C., 20 hrs., supernatant siphoned off, precipitate-suspension filtered on No. 3 paper.... Precipitate....	13				
<i>No. 13</i> + 2 liters 0.2 M pH 7.6 phosphate buffer.....	14	2200	20	90	40
2.2 liters <i>No. 14</i> + 550 ml. saturated ammonium sulfate (0.2 saturated) 25°C., 24 hrs., filtered on folded No. 3 paper..... Precipitate....	15P				
Precipitate-suspension <i>No. 12</i> centrifuged, precipitate + 600 ml. 0.2 M pH 7.6 phosphate buffer + 40 gm. standard Super Cel, filtered..... Filtrate.....	21	600	30		
Precipitate....	21P				
600 ml. <i>No. 21</i> + 150 ml. saturated ammonium sulfate (0.2 saturated) 24 hrs., 25°C., filtered on folded No. 3 paper..... Precipitate....	23P				

TABLE II—Continued

	No.	Vol.	Protein/ml.	Anti-body	Total AB
		ml.	mg.	per cent	gm.
No. 23P + 15P stirred with 1 liter 0.05 saturated ammonium sulfate, filtered by suction on 25 cm. No. 303 E and D paper..... Filtrate.....	24	1000			
1 liter No. 24 + 250 ml. saturated ammonium sulfate (0.25 saturated), stirred slowly at 25°C. 24 hrs. + 30 gm. standard Super Cel, filtered... Precipitate...	26P				
No. 26P + 1500 ml. 0.1 M pH 7.6 phosphate buffer, stirred, filtered, milky..... Filtrate.....	27				
Precipitate + 500 ml. 0.1 M pH 7.6 phosphate buffer, stirred, filtered..... Filtrate.....	28				
No. 27 + 28.....	29	2000	14	100	28
800 ml. No. 29* + 160 ml. saturated ammonium sulfate (0.17 saturated), stirred in slowly, flaky precipitate, filtered, slightly cloudy..... Filtrate.....	30	1000	9	95	9
Filtrate No. 30 + 10 gm. standard Super Cel, filtered by suction on 20 cm. No. 3 paper, bluish filtrate, stirred, and saturated ammonium sulfate added slowly until slightly cloudy, 5°C., 24 hrs., granular precipitate, decanted and centrifuged..... Precipitate...	41				
Supernatant..	42				
Precipitate No. 41 + 50 ml. water, nearly clear, filtered on No. 3 paper, stirred and saturated ammonium sulfate added until slightly cloudy, amorphous precipitate. 100 ml. water added, clear solution, saturated ammonium sulfate stirred in until slightly cloudy, filtered on No. 3 paper. Saturated ammonium sulfate added until slightly cloudy and stirred, granular precipitate, allowed to stand at 5°C. for 1 mo. Centrifuged, precipitate + 50 ml. water, clear, stirred, and saturated ammonium sulfate added until very slightly cloudy and stirred at 25°C. 2 days, good rosettes. Centrifuged, precipitate + 10 ml. water, nearly clear, saturated ammonium sulfate added until slightly cloudy, filtered, 10 ml. water added, stirred and saturated ammonium sulfate added until slightly cloudy, stirred at 25°C. 2 days, good rosettes	51	60	56	97	3
No. 42 stirred and saturated ammonium sulfate added slowly until slightly cloudy, stirred 24 hrs., granular precipitate, centrifuged, precipitate + 100 ml. water, nearly clear, stirred and saturated ammonium sulfate added until slightly cloudy, rods and rosettes, allowed to stand 1 mo. at 5°C.....	52				

TABLE II—*Concluded*

	No.	Vol.	Pro- tein/ml.	Anti- body	Total AB
		ml.	mg.	per cent	gm.
Diluted with equal volume of water, stirred 24 hrs., poor rosettes, filtered, precipitate dissolved in 40 ml. water, stirred and saturated ammonium sulfate added until slightly cloudy, filtered by gravity on No. 3 paper, bluish filtrate, stirred and inoculated with <i>No. 51</i> , stirred 24 hrs., poor rosettes.	521	50	18	95	.9
Centrifuged, precipitate + 30 ml. water, stirred and saturated ammonium sulfate added until slightly cloudy, stirred 24 hrs. at 25°C., large flakes and poor rosettes, centrifuged, precipitate + 30 ml. water, 5°C., 24 hrs., stirred and saturated ammonium sulfate added until slightly cloudy, 25°C., 24 hrs., heavy precipitate, good rosettes					

* The remainder of solution 29 was used in other fractionation procedures, which failed to yield crystalline material.

sulfate. A crystalline precipitate should form in an hour or so and most of the protein should be out of solution after 24 to 36 hours. Recrystallization is carried out in the same way. The crystals dissolve quite slowly and 24 hours' stirring is required to complete the solution. There is always more or less insoluble protein left, after the crystals have dissolved.

Preparation of Various Antibody Fractions

The method of preparing one sample of crystalline antibody is shown in outline in Diagram 1 and in detail in Table II. Crystalline material was obtained from four different lots of antibody solution and from three different lots of serum. The steps in the isolation varied somewhat in each case but in every case the crystalline material was obtained from the same fraction; that which precipitated in the range of from 0.17 to 0.2 saturated ammonium sulfate at pH 6 to 7. Some sera had a higher content of this fraction than did others so that no general figure for the total yield of crystalline material can be given. The antibody solution used in Experiment 152 (Table II) contained the highest proportion of the fraction and that reported in a subsequent experiment (Experiment 180, Diagram 2, not presented in table form) the lowest.

The fraction of antibody insoluble in neutral salts, and also the fraction precipitating at 0.17 saturated ammonium sulfate are mucilaginous and do not give clear solutions except in dilute acid, pH 3 to 4, or dilute alkali, pH 9.5 to 10.5. Precipitates of these fractions are extremely difficult to centrifuge or filter. If large amounts of filter aids, such as Filter Cel, are used, the filtration is fast, but most of the protein cannot be recovered from the Filter Cel. Filtration by gravity through folded No. 3 Whatman paper gives the clearest filtrate but is extremely slow. Centrifugation was carried out in an angle centrifuge at about 5,000 R.P.M.

Diagram 2
Fractionation Experiment 180

3.1 L. Type I pneum. AB soln. dil. to 18.6 with dist. H₂O,
24 hrs., 25°, decant and filter with hyflo

Ppt., stir with 2.5 L. 0.2 M NaCl, repeat, filter

Ft. B, 4.6 L. 60% AB + 1.6 L. 0.2 M pH 3.6 KH-phthalate, 25°,
24 hrs. + filter cell & hyflo & filter

Ft. I, 5.3 L. 95% AB + 1.3 L. SAS (0.2 sat) 5°, 24 hrs., decant
and cent.

Ppt. 2
+ 2 L. 0.2 M pH 7.6 PO₄ + SAS to 0.2 sat. 24 hrs., 25°, siphon
and filter with hyflo

Ppt. 13P
Extract twice with 1 L.
0.1 M pH 7.6 PO₄

Ppt. 40P
Sko + 2 L. 0.05 SAS ti-
trates to pH 10.5, filter

Ft. to pH 6.5, Ft. 42, 95%
AB + SAS to 0.15 sat.,
cent.

Sup. 43
+ 100 ml. 0.05 SAS, cent.
Sup. 43, 100% AB

Ppt. 41
+ SAS to 0.2 sat., cent.

Ppt. 45
+ 30 ml. 0.05 SAS, cent.

Sup. 50
+ SAS to 0.3 sat., 25°, 24 hrs., cent.

Ppt. + 200 ml. 0.05 SAS, cent.

Sup. 53
to pH 6.5, stir + SAS to 0.25 sat., stir, cent.

Ppt. + 50 ml. 0.05 SAS, cent.

Sup. 57, 90% AB
+ SAS till al. cloudy, stir 8 days 25°, good rosettes, cent.

Ppt. + 30 ml. 0.05 SAS, 5°, 24 hrs., al. amorphous ppt., cent.

Sup. to 6.5 + SAS till al. cloudy, stir 25°,
good rosettes at first becoming rounded, 59

Sup. 8
Add SAS to 0.33 sat. 24 hrs., 25°, cent.

Ppt. 10
+ 700 ml. 0.1 M pH 7.6 PO₄ + SAS to 0.2 sat., 24 hrs., 25°, decant and cent.

Sup. 14
1.2 L., stir & add 30 ml. SAS, gran ppt., cent.

Ppt. 14P
+ 900 ml. 0.1 M pH 7.6 PO₄ + SAS till al. cloudy,
stir 24 hrs., 25°, large flakes, cent.

Sup. 16
+ 200 ml. 0.05 SAS, filter

Ppt. 15P
Ft. + SAS till al. cloudy,
pH 6.5 stir 24 hrs.,
large flakes, cent.

Sup. 21
+ 200 ml. 0.05 SAS, filter

Ppt. + 200 ml. 0.05 SAS,
cent., Sup. 230, 75%

Sup. 25
Stir and add SAS till al. cloudy,
stir 48 hrs., rosettes and rods,
cent.

Ppt. + 100 ml. 0.05 SAS, cent.

Sup. 27
To pH 6.5, stir + SAS till al. cloudy,
good rosettes and rods, cent.

Ppt. + 50 ml. 0.05 SAS to pH 6.5, cent.

Sup. + SAS till al. cloudy, pH 6.5,
27 L., 100% AB

Sup. + SAS till al. cloudy, cent.

Ppt. + 50 ml. 0.05 SAS, cent.

Sup. + 1/4 vol. SAS, cent.

Ppt. + 50 ml. 0.05 SAS, cent. Sup. 225, 100%, stir, add 1/4 vol. SAS, cent.

Ppt. + 50 ml. 0.05 SAS, al. cloudy, cent., sup. to pH 6.5 + SAS till al. cloudy, filter

Ft. stir 25°, 4 days, fair rosettes, some large flakes, cent.

Ppt. + 40 ml. 0.05 SAS, al. cloudy, filter

Ft. to pH 6.5 + SAS till al. cloudy, stir 25°, good rosettes at first,
becoming rounded, 232, 95%

Preparation of Dissociated Antibody and Solutions of Antibody Used in Immunological Tests

The immune precipitate was prepared from the stock antibody solution used to prepare crystalline fraction 152-52. The method of preparation was the same as that described by Heidelberger and Kendall (1936) as modified by Goebel, Olitsky, and Saenz (1948). This preparation was 80 per cent precipitable by the homologous polysaccharide.

Stock solutions of the crystalline antibody were prepared as follows. Approximately 100 mg. of each of the crystalline antibody fractions, suspended in ammonium sulfate, was centrifuged at low speed, and the supernatant liquid decanted. The material was taken up in 5 ml. of 0.2 M sodium chloride and permitted to stand at room temperature for 6 hours, then overnight in the ice box. The substances were dialyzed free of SO_4^{2-} against a 0.2 M solution of sodium chloride containing 0.02 M sodium phosphate buffer at a pH of 7.2, and analyzed for their nitrogen content. Merthiolate was added so that the final concentration was 1 part in 20,000. These stock solutions were stable, and contained approximately 2 to 3 mg. of antibody nitrogen per ml. They were diluted to the appropriate concentration with sterile physiological salt solution prior to their use in the experiments described below.

Fraction No. 180-260 was brought into solution in the following manner. Approximately 2 ml. of the suspension of the immune protein was stirred with 50 ml. of 0.2 M phosphate buffer at pH 7.4. To this was added with stirring 2.0 ml. of M/1 sodium hydroxide. The final pH of the solution was 9.8. Part, but not all, of the protein dissolved. The material was dialyzed at 6°C. against successive changes of a 0.2 M phosphate buffer which had also been adjusted to pH 9.8, until free of ammonium sulfate. The solution was then analyzed for total nitrogen and then centrifuged after adjusting the pH to 7.8. Approximately 60 per cent of the total protein nitrogen remained in solution.

Comparison of the Immunological Properties of the Crystalline and Dissociated Antibody

1. *Agglutination of Types I and II Pneumococci.*—In order to determine the maximum dilution at which the crystalline antibody protein would agglutinate Type I pneumococci, serial dilutions of the solution of fraction 152-52 were prepared, and tested against a suspension of a 6 hour culture of washed Type I microorganisms killed by heating for 30 minutes at 60°C. 0.5 ml. of the appropriate antibody dilution in 0.9 per cent NaCl was mixed with 0.5 ml. of a suspension of pneumococci. The tubes were incubated at 37°C. for 2 hours, and after standing at 5°C. overnight, the agglutination reactions were recorded. For purposes of comparison a solution of the dissociated antibody was at the same time tested, as were the agglutination reactions of Type II pneumococci in solutions of both antibodies. The results recorded in Table III show that the minimum amount of crystalline antibody protein required to cause agglutination of Type I pneumococci was 8 micrograms of antibody nitrogen, whereas twice this amount of the dissociated antibody protein was necessary to cause comparable agglutination. It can also be seen from Table III that the agglutination was specific, for neither antibody agglutinated Type II pneumococci.

2. *Protective Action of Type I Pneumococcal Antibody Fractions.*—The protective power of the various crystalline and amorphous antibody protein fractions was tested by injecting 20 gm. Swiss mice intraperitoneally with a fixed quantity of virulent Type I pneumococci and varying quantities of the antibody proteins. 0.5 ml. of each antibody dilution, together with 0.5 ml. of a 6 hour culture of Type I pneumo-

TABLE III

Agglutination Reactions of Type I and II Pneumococci in Type I Crystalline Pneumococcal Antibody and in Dissociated Type I Antibody

Antibody fraction tested	Pneumococci type	Antibody nitrogen in sample tested, $\mu\text{g.}$					
		64	32	16	8	4	2
Fraction 152-52	I	++++	++++	++++	++	0	0
Dissociated antibody	I	++++	+++	++	0	0	0
Fraction 152-52	II	0	0	0	0	0	0
Dissociated antibody	II	0	0	0	0	0	0

TABLE IV

Protective Action of Pneumococcal Antibody Protein Fractions against 10^6 L.D. Type I Pneumococci

Antibody fraction tested	Antibody nitrogen injected, $\mu\text{g.}$									Antibody N protecting 50 per cent of mice
	40	20	10	5	2.5	1.25	0.62	0.31	0.16	
180- 69			5/6	6/6	2/6	0/6	1/6			$\mu\text{g.}$ 2.9
180-299			6/6	6/6	4/6	1/6	0/6			1.9
152- 52			6/6	5/6	3/6	0/6	0/6			2.8
180-260	5/6	5/6	6/6	2/6	2/6	0/6				6.3
Dissociated			6/6	6/6	6/6	6/6	4/6	0/6	0/6	0.5

Virulence controls: 10^{-6} 0/3

10^{-7} 0/3

10^{-8} 2/3

Plate count: 10^{-7} 12, 13 colonies

10^{-8} 4, 1 colonies

Numerator, survivals. Denominator, deaths.

cocci containing 2×10^8 microorganisms per ml., was injected into each of six mice. The virulence of the bacterial culture was such that 0.5 ml. of a 10^{-8} dilution, containing one to two microorganisms, killed mice when injected intraperitoneally. The animals were observed, after injection, for a period of 5 days and their deaths recorded in Table IV.

From the results presented in Table IV it can be seen that the amount of crystalline antibody protein nitrogen necessary to protect half the animals

against a million lethal doses of Type I pneumococci was approximately 2 to 3 micrograms. On the other hand, it required 6.35 micrograms of antibody nitrogen of the amorphous fraction No. 180-260² to afford comparable protection, whereas only 0.5 microgram of the dissociated antibody was necessary to give equal protection. It can also be seen in Table V that the protective action of the crystalline antibody is type-specific, for the protein affords no protection to mice infected with virulent Type II pneumococci.

In summarizing the results of the protection experiments it may be stated that the crystalline antibody fractions all had approximately the same protective action, based on the antibody nitrogen content, whereas the amorphous fraction No. 180-260 which formed by far the greater part of the antibody obtained during the process of crystallization was low in protective value. The dissociated antibody, on the other hand, was more potent than any of the fractions tested.

TABLE V

Protective Action of Crystalline Pneumococcal Antibody Fraction 152-52 against 10⁶ L.D. Type II Pneumococci

Antibody fraction tested	Antibody injected, μ g.			
	32	16	8	4
152-52	0/3	0/3	0/3	0/3
Virulence controls: 10 ⁻⁷ 0/3 10 ⁻⁸ 0/3				Plate count: 10 ⁻⁷ 28, 31 10 ⁻⁸ 4, 2

3. *Precipitin Reactions of Pneumococcal Antibody Fractions.*—In order to determine whether the various pneumococcal antibody fractions were immunologically related to the globulins present in normal horse serum, the fractions were tested in antiserum prepared by the immunization of rabbits with normal horse serum. Such an immune serum will contain antibodies directed against all the proteins present in normal horse serum. If pneumococcal antibody is a protein dissimilar to the proteins in normal horse serum, there should be no precipitin reaction when the test is performed. Preliminary experiments showed that all the fractions reacted in an antiserum to normal horse serum.

In order to determine whether differences in the intensity of the reactions could be observed, the precipitin tests were carried out quantitatively using a phototurbidimeter (Libby, 1938). Results of these experiments are represented graphically in Fig. 5.

²This fraction when first prepared was completely precipitable with the specific polysaccharide. At the time the above experiments were performed it was only 72 per cent precipitable.

From the results presented it can be seen that the crystalline fractions 152-52, 180-69, and 180-299 reacted all with nearly equal intensity, whereas fraction 180-260, the amorphous material, gave a reaction which was somewhat stronger. The amorphous dissociated immune globulin, on the other hand, gave a reaction the intensity of which was considerably lower than that of the crystalline proteins.

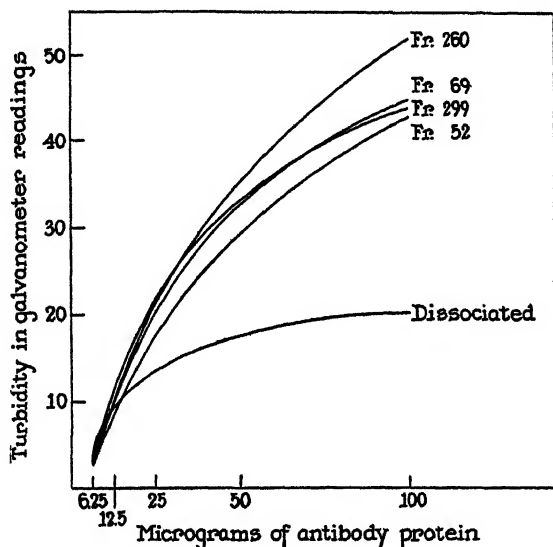


FIG. 5. Turbidimetric titration of pneumococcal antibody proteins in an antiserum to normal horse serum.

DISCUSSION

The present experiments show that it is possible to prepare a series of protein fractions from antipneumococcus horse serum, all of which are completely or nearly completely precipitated by the specific polysaccharide but which differ in their solubility, protective value, agglutinating power, and reaction with antihorse rabbit serum (Table VI). One of the fractions may be obtained in poorly crystalline form.

These results show quite conclusively that a whole series of proteins exists which precipitate specifically with the polysaccharide. It appears extremely unlikely, for instance, that a protein fraction which is completely insoluble in dilute neutral salts (fraction 180-260) could contain enough of a soluble protein, such as fraction 152-52, to cause the whole complex to precipitate completely with the polysaccharide. Whether the proteins exist as such in the serum or are modified during the fractionation is uncertain. The results also indicate that these immune proteins possess both protective and precipitating proper-

ties in varying proportion, as suggested by Heidelberger and Kendall (1935) and Goodner and Horsfall (1937).

The crystalline diphtheria antitoxin (Northrop, 1942) also protected against, as well as precipitated with, the toxin. This preparation was very nearly homogeneous and hence there is reason to think that in this case, at least, the same protein may possess both precipitating and protective properties.

This preparation did not precipitate with rabbit antihorse serum. This may be due to the fact that it was much more homogeneous than the present

TABLE VI
Summary of Properties of Various Antibody Preparations

Fraction	Solubility		Per cent precipitable + polysaccharide	Amount required to protect	Amount required to agglutinate	Reaction with rabbit antihorse serum
	Soluble in	Insoluble in				
Dissociate antibody	0.1 M NaCl	H ₂ O	80	μg. 0.5	μg. 16	+
152-52 Crystalline	0.1 M NaCl 0.15 saturated ammonium sulfate	H ₂ O 0.25 saturated ammonium sulfate; H ₂ O	95	2.8	8	++
180-69 Crystalline	" "	" "	90	2.9		++
180-299 Crystalline	" "	" "	95	1.9		++
180-260 Amorphous	pH 3-5 9-10	pH 5-9 All neutral salts	100* 70‡	6.3		+++

* When first prepared.

‡ When protective test was made.

preparations or because it was changed during the digestion of the immune precipitate with trypsin.

Unfortunately, none of the present fractions is even approximately homogeneous, as judged by the solubility test (Northrop, Kunitz, and Herriott, 1948), so that it is possible that the varying protective values are due to the presence of varying quantities of a special highly active protective antibody. A definite decision between the two possibilities probably cannot be made until a strictly homogeneous immune protein is prepared.

SUMMARY

1. The immune precipitate formed by antipneumococcus horse serum and the specific polysaccharide is not hydrolyzed by trypsin as is the diphtheria

toxin-antitoxin complex, and purified pneumococcus antibody cannot be isolated by the method used for the isolation and crystallization of diphtheria antitoxin.

2. Type I pneumococcus antibody, completely precipitable by Type I polysaccharide, may be obtained from immune horse serum globulin by precipitation of the inert proteins with acid potassium phthalate.

3. The antibody obtained in this way may be fractionated by precipitation with ammonium sulfate into three main parts. One is insoluble in neutral salts but soluble from pH 4.5 to 3.0 and from pH 9.5 to 10.5. This is the largest fraction. A second fraction is soluble in 0.05 to 0.2 saturated ammonium sulfate and the third fraction is soluble in 0.2 saturated ammonium sulfate and precipitated by 0.35 saturated ammonium sulfate. The second fraction can be further separated by precipitation with 0.17 saturated ammonium sulfate to yield a small amount of protein which is soluble in 0.17 saturated ammonium sulfate but insoluble in 0.25 saturated ammonium sulfate. This fraction crystallizes in poorly formed, rounded rosettes.

4. The crystallization does not improve the purity of the antibody and is accompanied by the formation of an insoluble protein as in the case of diphtheria antitoxin.

5. None of the fractions obtained is even approximately homogeneous as determined by solubility measurements.

6. Purified antibody has also been obtained by dissociating the antigen-antibody complex.

7. The protective value of the fractions is quite different; that of the dissociated antibody being the highest and that of the insoluble fraction, the lowest.

8. All the fractions are immunologically specific since they do not precipitate with Type II polysaccharide nor protect against Type II pneumococci.

9. All the fractions give a positive precipitin reaction with antihorse rabbit serum. The dissociated antibody gives the least reaction.

10. Comparison of the various fractions, either by their solubility in salt solution or through immunological reactions, indicates that there are a large number of proteins present in immune horse serum, all of which precipitate with the specific polysaccharide but which have very different protective values, different reactions with antihorse rabbit serum, and different solubility in salt solutions.

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